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Immunological and Developmental Factors Affecting the Efficacy of in Vitro Fertilization, Embryo Culture and Embryo Transfer in the Domestic Cat.

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IMMUNOLOGICAL AND DEVELOPMENTAL FACTORS AFFECTING THE EFFICACY OF *IN VITRO* FERTILIZATION, EMBRYO CULTURE AND EMBRYO TRANSFER IN THE DOMESTIC CAT

A Dissertation

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DEDICATION

This dissertation is dedicated to the endangered and threatened species of this planet that, lacking a voice of their own, need all the human advocates that they can find. These species have few options beyond their particular biological niches and, sadly, humans, the most adaptable of all species, seem to value self indulgence above morality. The extinction of many of these species is inevitable, to the impoverishment of the world.

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ABSTRACT

Developmental and immunological factors affecting the efficacy of assisted reproduction for domestic and endangered nondomestic felid species were investigated. In the first study, domestic cats exhibited decreased ($P<0.05$) numbers of follicles and maturity of oocytes following repeated treatments, at short intervals (44-50 days), with a combination regimen of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). Queens had elevated titers of eCG/hCG-binding immunoglobulins compared with male cats and naive queens, and these immunoglobulins attenuated eCG and hCG bioactivity in mouse ovarian stimulation assays. However, refractory queens appeared to cycle naturally and were responsive to an alternative exogenous gonadotropin, porcine follicle stimulating hormone (pFSH). In the second study, queens exhibited individual variability in immune responses to eCG and hCG, and this variability affected ($P<0.05$) subsequent ovarian responses. Ovarian follicular responses of queens did not decrease ($P>0.05$) when longer intervals (130-135 days) were used between successive eCG/hCG treatments. In the third study, the effects of eCG and hCG on the suitability of the maternal environment were investigated. Following intramuscular injection, eCG had an elimination half-life of 24 to 48 hours and, 5 and 15 days after follicular aspiration, eCG/hCG-treated queens had formed multiple ancillary follicles and secondary corpora lutea (CL), respectively. Treatment with antisera against eCG/hCG slowed ($P<0.05$) primary CL formation but did not alter ($P>0.05$) serum progesterone levels or decrease ($P>0.05$) the number of secondary ovarian structures. In the fourth study, developmental requirements of IVF-derived cat embryos were examined, with the emphasis on energy sources, media complexity and tissue co-culture and temporal variations in these factors. Embryos developed equally well ($P>0.05$) to morulae in simple media containing either glucose or glutamine, but blastocyst formation and embryo cell number were decreased ($P<0.05$) when glucose was present during the early culture period. Co-culture of embryos on oviductal cell monolayers decreased ($P<0.05$) fertilization and blastocyst

formation. In the final study, a transcervical technique for embryo transfer was developed and used successfully to produce viable offspring in the domestic cat.

INTRODUCTION

In a sense, the biological diversity of this planet is under siege (Wilson, 1988). An expanding human population, driven by egocentrism and lacking a long-term ecological perspective, have, through habitat destruction, pollution and direct predation, imperiled the very existence of scores of biological organisms. Estimations for the rate of species extinction vary, depending upon the particular assumptions in the calculations, but, in general, the consensus places the current rate of extinction at 1,000 - 10,000 times greater than the historical background level, prior to human intervention (Wilson, 1988). Among the 4000 species of mammals, felids have been among the most severely affected by this biological crisis. By their nature, cat species are highly sensitive to environmental pressures. As predators, cats require relatively large territorial areas (depending upon prey densities) and stable intact ecosystems. With human encroachment, both the reduction of territorial range and the disruption in the availability of traditional food sources increases the probability of direct confrontations between endemic cat populations and man. The introduction of livestock into the historic ranges of the larger cat species (such as the puma, jaguar and tiger) heightens the likelihood of such conflict (Seidensticker and Lumpkin, 1991). In addition, cat species have been particularly impacted by the whims of human fashion. Demands for spotted furs contributed significantly to the decimation of a number of cat species, primarily the larger cat species in the 1960s and early 1970s and many smaller cat species in the last 20 years (Fitzgerald, 1989). Because of these pressures upon their populations, of the 37 species of cats, all except the domestic cat have been listed as threatened or endangered by the Convention on the International Trade in Endangered Species (CITES), and 16 entire species and 12 other subspecies have been included on the U.S. endangered species list (Shoemaker, 1985).

The preservation of these cat species depends primarily on limiting habitat destruction in addition to strictly controlling international commercial trade of these animals. Unfortunately, global economic and social realities severely compromise the

potential effectiveness of such measures. The continued existence of many of these species ultimately will be determined by our ability to maintain genetically diverse populations in captivity. The captive propagation of any endangered species, however, faces inherent difficulties, dictated by both logistical and biological constraints. Although zoos presently breed about 19% of known mammalian species, few of the most severely endangered species of the world are being propagated in captivity (Conway, 1986). Captive populations of endangered species are frequently derived from a limited founder stock and have been subjected to extensive inbreeding. Inbreeding in zoo species has been associated with increased juvenile mortality (Ralls et al., 1979; Ralls and Ballou, 1982) and other deleterious effects such as reproductive failure and lowered disease resistance (Wildt, 1986; Wildt, 1990). Because zoos have a restricted financial and physical capacity to maintain individuals of a given species, most individual animals, depending upon prior representation in the gene pool, should be participants in the effective breeding population. The stated goal of the zoo community is to maintain 90% of the present genetic diversity for a period of 200 years. To obtain this goal, captive breeding programs must be managed to maximize the contribution of every individual within these populations (Benirschke, 1985; Conway, 1986; Seal, 1986).

Captive breeding programs have classically been restricted to the natural breeding of resident animals with occasional breeding loans from other zoological parks to limit inbreeding. With the changing realities of the current biological crisis, this strategy, while still of major importance, is no longer optimal for the propagation of all individuals and/or species. In many instances, due to behavioral or physical incompatibilities, reproductive insufficiencies and regulatory and/or logistical constraints, the natural breeding of animals cannot be consummated. In such situations, assisted reproductive technology may offer the only potential recourse to an otherwise permanent infertility. Assisted reproductive techniques, such as electroejaculation, superovulation, artificial insemination (AI), *in vitro* fertilization (IVF), embryo culture, embryo transfer (ET) and gamete/embryo

cryopreservation, are essential for the breeding management of endangered species. When fully developed, these techniques will facilitate genetic exchange between different captive populations and, eventually, between captive and wild populations (if these still exist). Ultimately, with the use of cryopreservation for genome resource banking, the concept of a "frozen zoo" may become a reality (Dresser, 1990; Wildt, 1992) and the long-term maintenance of genetic diversity may finally be assured for many species.

These assisted reproductive techniques are now commonly applied to alleviate certain failures in human reproduction and to enhance the production and/or selective breeding of domestic hoofstock, poultry and companion animals. The routine use of these procedures is the culmination of years of research into basic reproductive parameters and the investment of substantial economic capital. The direct extrapolation of these techniques to the reproduction of nondomestic species sounds attractive but is unrealistic given our limited knowledge of basic physiological norms in nondomestic species and the probability of species-specific differences in certain reproductive processes. Comparative studies, using domestic species as models for representative endangered species, are conducive for generating a closer approximation of optimal conditions for that particular species (Seager, 1983; Wildt and Bush, 1984; Wildt et al., 1986). For the nondomestic ungulate species, research with domestic cattle and sheep has fostered the successful extension of some procedures (Pope and Dresser, 1991; Schiewe et al., 1991). Similarly, research using the domestic cat as an animal model has broadened our knowledge of felid reproductive physiology and enhanced the suitability of assisted reproductive techniques for rare cat species (Wildt et al., 1980a; Goodrowe et al., 1988a; Pope et al., 1989a; Howard et al., 1992a).

Viable offspring of nondomestic cat species have recently been produced using two different strategies: 1) IVF of oocytes followed by embryo culture and transfer of oviductal or uterine stage embryos (Pope et al., 1989b; Donoghue et al., 1990) and 2) laparoscopic intrauterine AI (Howard et al., 1991b; Wildt et al., 1992b; Donoghue et al., 1993a). These

successes are encouraging but the overall efficacy of these procedures will require substantial improvement before broader application is possible. To reach this goal, more information is needed about basic physiological processes and responses in cats and a number of potential problems with assisted reproduction must be addressed.

Of major concern, assisted reproductive procedures require the administration of exogenous gonadotropins (equine chorionic gonadotropin, eCG; porcine follicle stimulating hormone, pFSH; human chorionic gonadotropin, hCG) to induce ovarian follicular development and ovulation in oocyte donors, AI recipients and/or embryo recipients. The immunological consequences of injection of these foreign proteins have been well established in other species (Willett et al., 1953; Lin and Bailey, 1965; Greenwald, 1970; Ottobre and Stouffer, 1985; Bavister et al., 1986) but have not been investigated in cats. The development of immunological refractoriness to repeated ovarian stimulation would limit the usefulness of these protocols in endangered cats and may even interfere with natural reproductive processes in these animals. If this occurs, however, changes in the frequency of gonadotropin stimulation or the use of alternative stimulation protocols could potentially mitigate or prevent these adverse effects. A second major concern is that exogenous gonadotropins may generate an inappropriate oviductal or uterine environment for the fertilization of oocytes or the development of embryos. In addition to potentiating undesirable immune responses, prolonged persistence of exogenous gonadotropins in circulation may overstimulate ovarian follicular and luteal development and alter the proper hormonal balance required for a suitable maternal environment (Dieleman et al., 1993). In this event, strategies to neutralize circulating gonadotropins, such as the passive transfer of anti-gonadotropin immunoglobulins, may be needed.

A third concern with the use of exogenous gonadotropins and assisted reproductive procedures in cats is that many of the resulting embryos may not be developmentally competent. Most cat embryos, generated from IVF, exhibit a developmental arrest (or block) at the morula stage during *in vitro* culture (Johnston et al., 1991a). In other species,

these blocks have been resolved by alterations in media complexity or energy sources or by co-culture with oviductal cells (Rexroad and Powell, 1988; Chatot et al., 1989). Similar approaches with cats may be beneficial for further defining the culture requirements of cat embryos and for assessing the viability of embryos produced through IVF. Another potential benefit of these culture studies could be to promote the development of noninvasive ET methods.

A fourth concern with assisted reproductive procedures in cats is that invasive, surgical manipulations (laparoscopy or laparotomy) are required for oocyte retrieval, semen deposition and ET. However, at least for AI and ET, a nonsurgical, transcervical approach could be a possible alternative. Because nonsurgical ET would require uterine stage embryos (morulae and blastocysts), successful culture of IVF embryos to these later stages would be a prerequisite. But, if feasible, transcervical ET would avoid many of the potential complications of surgery. Ultimately, however, the efficacy of nonsurgical ET, as with surgical ET, would still be dependent on the biological competence of IVF embryos and the suitability of the maternal environment to support pregnancy.

In this dissertation, these four specific concerns associated with IVF, embryo culture and ET in cats are investigated as part of a continuum in one reproductive strategy. As part of this continuum, these immunological and developmental concerns are interrelated, with complications affecting one process necessarily impacting on the efficacy of the strategy as a whole. In Chapter I, the pertinent literature is reviewed with the emphasis upon the normal reproductive physiology of domestic cats and the use of assisted reproductive techniques in domestic cats and other felid species. In Chapter II and III, the immunological consequences of exogenous gonadotropin - induced ovarian stimulation are addressed with particular attention given to alternative approaches to avoid potential immunological pitfalls. In Chapter IV, the effect of exogenous gonadotropins on the maternal environment and the impact of gonadotropin neutralization are examined. In Chapter V, the developmental factors influencing the culture and competency of IVF

embryos are addressed. Lastly, in Chapter VI, the feasibility on transcervical embryo transfer and factors affecting its efficacy are evaluated.

CHAPTER I

LITERATURE REVIEW

Natural Reproduction in the Domestic Cat

Reproductive Anatomy

The reproductive anatomy of the female domestic cat, or queen, is similar to that of other cat species (Wildt et al., 1980a) and to multiparous carnivore species in general. The ovaries, approximately 8-9 mm in length, are located just caudal to the kidneys and are attached cranially by the suspensory ligament and dorsally by a portion of the broad ligament, the mesovaria. The mesosalphinx envelops the oviducts and covers the lateral aspect of the ovaries to form the ovarian bursa. The oviducts, 4-5 mm in length, course cranially in the medial aspect of the ovarian bursa and then turn sharply to run caudally to the lateral aspect before terminating at the uterotubal junction as a small papillae. The uterus is bicornuate with the uterine horns, 9-10 cm in length and 3-4 mm in thickness, suspended dorsally by the mesometrium. The uterine body is 2 cm long and partially divided internally by a short septum. The cervix is very short and opens obliquely at the external orifice of the vagina (Crouch, 1969; Nickel et al., 1979).

The cranial vagina forms a ventral and lateral fornix around the external cervical orifice and possesses a dorsal median postcervical fold, similar to that described for the domestic dog (Pineda et al., 1973). The vagina extends caudally to the opening of the urethra on the ventral aspect at the vaginovestibular junction. The vestibule possesses glandular tissues laterally and terminates at the vulva. The vagina and vestibule combined are approximately 4 cm in length (Crouch 1969; Nickel et al., 1979; Watson and Glover, 1993).

The reproductive anatomy of the male domestic cat, or tom, is typical of other carnivore species with a few notable differences. The testes of the tom are descended at birth and are located in the scrotum caudodorsal to the penis (Scott, 1970). The penis, approximately 21 mm in length and 5 mm in width (Watson and Glover, 1993), is directed

caudally and is covered with 100-200 cornified papillae or spines. These papillae are testosterone-dependent and will regress in size following castration of the tom (Aronson and Cooper, 1967). The papillae may function to increase vaginal stimulus during coitus for the induction of ovulation in the queen (Herron, 1986). The distal portion of the penis contains a short (5 mm) os penis or baculum. Accessory reproductive organs in the tom consist only of paired bulbourethral glands and a prostate gland with both discrete and disseminated components (Crouch, 1969; Nickel et al., 1979).

Sexual Maturity and Seasonality

Male cats usually become sexually mature before 1 year of age at a minimal body weight of 3.5 kg (Scott, 1970). Spermatogenesis commonly begins within 8 months of birth, with sperm cell production apparently not affected by season (Kirkpatrick, 1985; Sanchez et al., 1993). The female domestic cat usually reaches puberty between 6-9 months of age (Scott, 1970; Jemmet and Evans, 1977) with some dependence upon season of birth and growth rate. Typically, queens weigh 2.3-2.5 kg at puberty (Scott, 1970). Queens are seasonally polyestrous, exhibiting seasonal patterns in reproductive cyclicity with the onset of estrous activity usually by February or March and with cycles extending until September (Jemmet and Evans, 1977; Herron, 1986; Schmidt, 1986). Because seasonality is affected by the length of the photoperiod, geographic location (i.e., latitude) has a strong influence on the degree of seasonality. Artificial lighting, of at least 12 hours per day, may be used to maintain cyclicity throughout the year (Wildt et al., 1978a; Hurni, 1981).

The Estrous Cycle

Proestrus

The estrous cycle of the queen has four defined phases: proestrus, estrus, diestrus (pseudopregnancy) and anestrus (or interestrus); each associated with specific changes in endocrine and behavioral characteristics and, to a lesser degree, in vaginal cytology (Herron, 1977; Wildt et al., 1978a; Shille et al., 1979; Feldman and Nelson, 1987).

Proestrus is defined behaviorally as the period preceding estrus in which the queen is sexually attractive to the male but will not permit coitus. The queen will often rub her head and neck against objects, vocalize frequently and roll over on her back (Michael, 1961). This phase is of short duration (≤1 day) and is infrequently observed. In one study, proestrus was seen in only 27/168 cycles and averaged just 1.2 ± 0.8 days in length (Shille et al., 1979). This period is associated with an increase in follicular size and estradiol levels (<20 pg/ml) (Foster and Hisaw, 1935; Shille et al., 1979). The ovaries possess indistinct dark areas, about 1 mm in diameter, on their surface that are destined to form pre-ovulatory follicles (Dawson and Friedgood, 1940; Wildt and Seager, 1980). With the rising estradiol levels, vaginal cytology may show a slight increase in cornification of epithelial cells but, more typically, a clearing of noncellular debris on the vaginal smear may be seen (Mowrer et al, 1975; Shille et al., 1979).

Estrus

Following the short proestrus phase, queens will usually enter the estrous phase, a period characterized behaviorally by the queen permitting mounting and coital activity. Other behavior may include intense vocalization, frequent urination and and the assumption of lordosis (i.e., bent forelegs, elevated pelvis and deviated tail). Queens may also "tread" their hind feet in response to stimuli. These behaviors may be elicited by mounting activity or by stroking of the flanks and perineal region by a handler (Michael, 1961). Generally, the exhibition of estrous behavior follows an increase in estradiol levels associated with follicular development. Estradiol levels rise rapidly from a basal plasma level of about 15 pg/ml, just prior to the onset of behavioral estrus, to levels >20 pg/ml during sexual receptivity, but individual estradiol concentrations reportedly fluctuate considerably during estrus (Wildt et al., 1981). Peaks in estradiol concentrations during estrus can vary from 25-80 pg/ml of plasma (Shille et al., 1979; Wildt et al., 1981).

The exhibition of estrous behavior lags behind the increase in plasma estradiol concentrations with only 8% of cats showing estrus on Day 1 of the follicular phase (>20

pg/ml) but 80% showing such behavior by Day 4 (Shille et al., 1979). At laparoscopy, distinct vesicular follicles (≥2 mm in diameter) are seen protruding slightly above the ovarian surface (Wildt and Seager, 1980). In a typical cycle, queens possess 3-7 follicles on both ovaries (Foster and Hisaw, 1935; Wildt et al., 1981). The follicular phase has been reported to be 7.4 ± 2.3 days in length with coitus and/or ovulation not affecting the duration of elevated estradiol levels. The effect of coital activity upon the duration of estrus behavior, however, is less well-defined with contradictory results indicating that coitus is associated with a decreased length of estrus (Scott and Lloyd-Jacob, 1955), an increased length of estrus (Shille et al., 1979) or no change in estrus length (Wildt et al., 1981). Differences between studies were attributed to alternative breeding protocols with the timing of mating within the estrous phase as a possible factor (Wildt et al., 1981). In the absence of mating, the length of the estrous phase was similar between studies, ranging from a mean duration of 6.4 days (Wildt et al. 1981) to 7.2 days (Shille et al. 1979).

Female domestic cats are considered to be induced ovulators (Greulich, 1935; Dawson and Friedgood, 1940), although spontaneous ovulation has been reported (Hill and Tribe, 1924; Dow, 1962; Lawler et al., 1993). Usually, however, the specific phase of the cycle subsequent to estrus is dependent upon the occurrence and intensity of coital activity. If the queen is not mated during estrus, she will gradually enter into an anestrous (or interestrous) phase, characterized behaviorally by her indifference or active resistance to sexual advances by the tom (Michael, 1961). In the absence of coitus, the mature ovarian follicles undergo atresia and plasma estradiol levels decrease to pre-estrual levels (<20 pg/ml). Vaginal cytology reflects this drop in estradiol with an increase in nucleated epithelial cell types and in noncellular debris on the stained smear (Herron, 1977; Shille et al., 1979). The interestrous phase averaged 8.1 days in one study (Shille et al., 1979) but this period varies considerably between individual queens, with ranges of 3-15 days reported (Jemmet and Evans, 1977; Wildt et al., 1978a; Shille et al., 1979). However, the

interval between estrous phases was unaffected by coital activity if ovulation did not ensue (Shille et al., 1979).

Induced Ovulation

If coitus occurs, the queen can ovulate and enter into a diestrus or luteal phase, such as with pregnancy or, if the mating is infertile, pseudopregnancy (Paape et al., 1975; Wildt et al., 1981). The induction of ovulation is dependent upon a neuroendocrine pathway involving coitus-induced neuronal stimulation of the medial basal hypothalamus (Robinson and Sawyer, 1987), a reflex release of gonadotropin-releasing hormone (GnRH) and a subsequent pulse of luteinizing hormone (LH) secretion from the pituitary gland (Concannon et al., 1980; Wildt et al., 1981). With unrestricted or frequent mating during early estrus, LH levels surge from a basal level <4 ng/ml to greater than 70 ng/ml within 8 hours of coital activity and then gradually decline over the next 20-24 hours to precoital concentrations (Concannon et al., 1980; Wildt et al., 1981; Shille et al., 1983). This surge in LH is usually sufficient to cause final follicular maturation with ovulation reportedly occurring at variable timepoints post-first breeding, depending primarily upon the onset of mating within the estrous cycle.

While most queens mated beginning on the first day of behavioral estrus require at least 48 hours to ovulate (Wildt et al., 1981), cats mated initially on the second day of estrus are estimated to ovulate 30-36 hours later (Swanson et al., 1994e) and queens mated starting on the third or fourth day of estrus reportedly complete ovulation by 32 hours post-mating (Shille et al., 1983). Differences in follicular maturity during the days of early estrus (Donoghue et al., 1993b), and subsequent responsiveness to the LH surge could account for these variable ovulation intervals (Wildt et al., 1981; Shille et al., 1983; Wildt, 1991). This theory is consistent with evidence that estradiol heightens the ability of the hypothalamus and/or pituitary to generate an LH surge in response to coital activity (Banks and Stabenfeldt, 1982; Glover et al., 1985)

Ovulation appears to be an all-or-none phenomenon since the number of ovarian follicles (>2 mm in diameter) observed prior to mating corresponds to the number of CL seen post-mating (Wildt et al., 1980a). However, both the amplitude and duration of the rise in LH levels may affect the ultimate outcome (Chakraborty et al., 1979; Wildt et al., 1980b). If mating activity is limited to a single copulation, an LH surge frequently does not occur and ovulation is prevented (Concannon et al., 1980; Wildt et al., 1980b; Johnson and Gay, 1981; Glover et al, 1985). This neuroendocrine pathway is subject to fatigue with copulations on days following an LH surge associated with decreased LH surges relative to the previous day (Wildt et al., 1981). Similarly, with unrestricted copulations on a given day, the later copulations will produce no further increase in LH levels. The reflex release of LH is self-limiting with the suppression of the coitus-induced LH surge persisting as long as 48-72 hours (Concannon et al., 1989). However, postcoital after-reactions of the queen are not inhibited and injection of physiological doses of GnRH may induce a pronounced spike in LH levels, indicating that the decreased responsiveness is probably caused by depletion of hypothalamic GnRH levels or feedback inhibition of GnRH on further GnRH release from the hypothalamus (Concannon et al., 1989). Although considerable research has focused on LH secretory patterns in cats, the nature and pattern of FSH release has not been investigated (Goodrowe et al., 1989a).

Diestrus (Pseudopregnancy)

If ovulation occurs in response to coitus, the queens enter diestrus, a phase dominated by elevated progesterone levels. Behaviorally, the queen no longer displays estrual postures and is generally unresponsive to the male, as in anestrus (Michael, 1961) and parabasal cells are more commonly seen in the vaginal cytology (Herron, 1977; Shille et al., 1979). Following mating, vesicular ovarian follicles (2.5-3.5 mm in diameter) become more vascularized, develop a central hyperemic stigma and release the follicular contents onto the ovarian surface, with a slight inward collapse of the follicular apex (Dawson and Friedgood, 1940; Wildt and Seager, 1980a; Shille et al., 1983) Within 24-48

hours of ovulation, corpora lutea develop as raised, reddish-orange structures, reaching their maximum size (3.6-4.5 mm) by 10-16 days post-ovulation (Dawson, 1946; Wildt and Seager, 1980; Wildt et al., 1981). In pseudopregnant queens, coincidentally with CL development, progesterone levels increase from basal concentrations of <1 ng/ml (Paape et al., 1975; Wildt et al., 1981; Schmidt et al., 1983) to peak levels of 25-90 ng/ml by 14-22 days post-ovulation (Paape et al., 1975; Verhage et al., 1976; Wildt et al., 1981). Unlike in dogs (Concannon et al., 1977), ovulating queens do not exhibit a pre-ovulatory rise in progesterone so the first measurable increase in serum progesterone levels is relatively delayed (Wildt et al., 1981), and queens in pseudopregnancy do not show any of the physical or behavioral changes (mammary development, nesting) typically seen in pseudopregnant dogs (Herron, 1986).

The duration of pseudopregnancy (period of progesterone concentrations >1 ng/ml) has been reported at 36-38 days (Paape et al., 1975; Shille and Stabenfeldt, 1979; Wildt et al., 1981), with the CL gradually regressing, beginning in the mid-luteal phase, to form a persistent yellow luteal scar (Wildt and Seager, 1980; Wildt et al., 1981). Following the end of pseudopregnancy, queens usually begin a new estrous cycle within 7-14 days so that the interestrous interval (with sterile mating) typically is 40-50 days in duration (Paape et al., 1975; Wildt et al., 1981). Interestingly, premature regression of the cat CL cannot be induced by treatment with prostaglandin $F_{2\alpha}$ (Shille and Stabenfeldt, 1979; Wildt et al., 1979a), although a slight depression in plasma progesterone levels may result with treatment in late pseudopregnancy (Shille and Stabenfeldt, 1979). Similarly, premature luteal regression cannot be induced by hysterectomy during early pseudopregnancy (Wheeler et al., 1988). Prostaglandin treatment may cause abortion if administered in late pregnancy (>40 days) (Nachreiner and Marple, 1974) but this effect is probably mediated more through uterine smooth muscle contractility than luteolysis. In general, little information is available concerning the physiology of CL function in cats, especially

regarding luteal cell types, luteal hormone receptors or putative luteotrophic and luteolytic substances.

Pregnancy

Endocrinology

Endocrine profiles during pseudopregnancy and pregnancy are similar during the early luteal period (Verhage et al., 1976; Schmidt et al., 1983), but progesterone concentrations may peak slightly later (>20 days post-ovulation) and at a higher level in pregnancy than with pseudopregnancy (Verhage et al., 1976). In addition, the duration of pseudopregnancy is considerable shorter (~36 days) than that of pregnancy (63-67 days) (Verhage et al., 1976; Jemmet and Evans, 1977; Schmidt et al., 1983). However, progesterone levels during pseudopregnancy and pregnancy show a high degree of individual variation (Schmidt et al., 1983) so, until ~40 days post-ovulation, progesterone levels cannot be used as a reliable indication of pregnancy status (Schmidt et al., 1983). During pregnancy, progesterone levels decline slowly beginning ~30 days into pregnancy and reach basal levels on or shortly following the day of parturition (Verhage et al., 1976; Schmidt et al., 1983). During the first 40-45 days of pregnancy, progesterone is produced primarily by the CL, and because ovariectomy at this time does not induce abortion (Scott, 1970), the placenta has been credited with progesterone production during the last three weeks of pregnancy (Malassine and Ferre, 1979). However, recent evidence suggests that the CL is required throughout pregnancy in the cat and that the placenta plays a relatively minor role in progesterone secretion (Verstegen et al., 1993a).

During the second half of pregnancy, estradiol concentrations may fluctuate slightly above basal levels, possibly indicating ovarian follicular development, and queens frequently exhibit a estradiol surge 8-9 days prior to parturition (Verhage et al., 1976; Wildt et al., 1981; Schmidt et al., 1983). Queens occasionally have been reported to mate during pregnancy (Festy and Bleby, 1970; Tsutsui and Stabenfeldt, 1993) with superfetation a possible but infrequent outcome (Markee and Hinsey, 1935).

A few studies have examined the changes in prolactin, relaxin and prostaglandin $F_{2\alpha}$ concentrations during pregnancy. Prolactin levels have been shown to increase about the 35th day of pregnancy and reach a plateau around 2 weeks later. Elevated prolactin levels are maintained through parturition and the first 4 weeks of lactation, before decreasing to basal concentrations shortly after weaning (Banks and Stabenfeldt, 1982; Tsutsui and Stabenfeldt, 1993). Limited evidence indicates that prolactin may be luteotrophic in cats during later pregnancy, since administration of an antiprolactin agent (cabergoline) at 30 or 40 days of pregnancy reduces progesterone levels to baseline within 72 hours and induces abortion in treated queens (Jochle et al., 1989; Verstegen et al., 1993a). Prolactin also may be responsible for delayed cyclicity post-partum since queens nursing young typically exhibit basal hormone levels and minimal ovarian follicular development (Schmidt et al., 1983). Relaxin levels also increase in cats beginning around day 25 of gestation to reach a plateau 5-10 days later but concentrations decline gradually to basal levels by the time of parturition (Stewart and Stabenfeldt, 1985). Similarly, prostaglandin $F_{2\alpha}$ production is reported to increase around day 30, plateau at day 45 and then exhibit a $PGF_{2\alpha}$ spike just prior to parturition before decreasing post-partum (Tsutsui and Stabenfeldt, 1993).

Embryo and Fetal Development

Until recently, little information was available concerning the early *in vivo* development of domestic cat embryos. Earlier studies described the morphological traits of ovarian follicles, cat oocytes and early-stage embryos (Hill and Tribe, 1924; Dawson and Friedgood, 1940) but provided little data on embryo development *in vivo* relative to time of ovulation. At the time of ovulation, the cat oocyte is in metaphase II of meiosis and ~95 μm in diameter (including the zona pellucida) (Hill and Tribe, 1924), although a recent oocyte measurement of ~160 μm in diameter is probably more accurate (Goodrowe et al., 1988a). The oocyte is fertilized within the upper third of the oviduct (van der Stricht, 1911) with the time of the first cleavage division relative to fertilization unknown *in vivo*,

but estimated at 24-30 hour post-fertilization *in vitro* (Bowen, 1977; Goodrowe et al., 1988a). *In vivo* embryo development was reported by Denker et al. (1978a) for a very limited number of cats but this study was confounded by the injection of queens with hCG or hCG/eCG to ensure ovulation and superovulation, respectively. Other limited studies (Herron and Sis, 1974; Goodrowe et al., 1988b) have indicated that cat embryos enter the uterus by 6 days post-copulation as morulae or blastocysts.

A more substantive study (Swanson et al., 1994e) has characterized embryo development *in vivo* and indicated that cat embryos exhibit a biphasic rate of growth within the oviducts, with rapid development to the 5- to 8-cell stage followed by slower cleavage to the morula stage. Cat embryos undergo compaction at the morula stage and transverse the uterotubal junction as compact morulae or early blastocysts between 124 and 148 hours post-mating (Swanson et al., 1994e). Within the uterus, cat embryos migrate extensively between uterine horns (Hill and Tribe, 1924; Tsutsui et al., 1989a; Swanson et al., 1994e) to evenly distribute the fetuses and presumably optimize fetal survival. Embryos escape from their zona pellucida by zonal lysis at ~12 days post-mating and begin implantation into the endometrium between days 13 and 14 post-mating (Denker et al., 1978b). Cats are reported to have a zonary, endotheliochorial type placentation with a simple crosscurrent relationship for materno-fetal blood flow (Leiser and Koob, 1993).

Pregnancy failure in cats, caused by fertilization failure, early embryonic mortality and/or implantation failure, has been reported to range from 16-33%, based on the ratio of the number of fetuses or kittens to the number of CL observed on the ovaries (Schmidt et al., 1983; Tsutsui et al., 1989a; Swanson et al., 1994e). Similar percentages (~30%) of embryo loss were observed with pre-implantation embryos, suggesting that implantation failure was not a significant cause of pregnancy loss (Swanson et al., 1994e). Both the mating strategy and the age and/or parity of the queen may affect the frequency of pregnancy failure (Schmidt, 1986; Swanson et al., 1994e).

Fetal growth is rapid, increasing from a crown-rump length and body weight of 1.7-2.2 cm and 0.5-1.1 g, respectively, at 24 days of age to 9.2-9.7 cm and 78.3-86.5 g at 51 days. At birth, kittens are 10-11 cm in length and weigh 85-105 g (Tsutsui and Stabenfeldt, 1993). The diagnosis of pregnancy and monitoring of fetal growth and health during pregnancy may be accomplished effectively with ultrasound examinations (Davidson et al., 1986). The gestation period of the domestic cat is 63-67 days in length (Verhage et al., 1976; Jemmett and Evans, 1977; Schmidt et al., 1983; Tsutsui and Stabenfeldt, 1993), and litter sizes usually average 4-5 kittens (Robinson and Cox, 1970; Jemmett and Evans, 1977).

Assisted Reproduction in Domestic and Nondomestic Felids

Semen Collection

The collection of semen from cats is a necessary prerequisite for most other assisted reproductive procedures, including artificial insemination, *in vitro* fertilization and sperm cell cryopreservation, and requires efficient, minimally traumatic methods of recovery. Semen may be collected by one of three methods: 1) artificial vagina (Sojka et al., 1970), 2) electroejaculation (Platz and Seager, 1978; Dooley et al., 1983; Wildt et al., 1983) or 3) post-mortem or post-castration recovery (Bowen, 1977; Goodrowe and Hay, 1993). The use of an artificial vagina (AV) is the most atraumatic technique of semen collection for domestic cats and allows the recovery of semen with volumes of ~30-40 µl and ~60 x 10^6 total sperm/ejaculate (Sojka et al., 1970; Platz et al., 1978). Cats may be collected by AV three times per week with no diminution in total sperm recovery or increase in abnormal sperm forms (Sojka et al., 1970). However, for this technique, toms must tolerate the presence of a handler and must be amenable to AV training, and many (~40%) domestic cats fail to adapt to the procedure (Sojka et al., 1970). Of concern, semen collection with an AV is generally not applicable to nondomestic cat species, although some hand-raised cheetahs (Durrant et al., 1992) have proven trainable to an AV. However, for conservation

purposes, a collection method that is more suitable for the entire felid population is essential.

Electroejaculation of anesthetized domestic cats permits the recovery of a more dilute, voluminous ejaculate (~100-200 µl) than with an AV but containing slightly less numbers of total spermatozoa/ejaculate (~30 x 10^6) (Platz et al. 1978; Wildt et al., 1983). Sperm motility (50-90%) and percentage of normal sperm forms (60-90%) are unaffected by collection method (Sojka et al., 1970; Platz et al., 1978; Wildt et al., 1983). Using a sine-wave electrostimulator and a three electrode rectal probe (Platz and Seager, 1978; Dooley et al., 1983), electrical stimulation of parasympathetic and sympathetic nerve fibers usually induces erection and ejaculation in both domestic and nondomestic cats (Howard et al., 1984; Wildt et al., 1988). In domestic cats, the seminal fluid recovered following electroejaculation has an osmolarity of ~323 mOsm/l with the bulbourethral glands contributing most of this fluid (Johnston et al., 1988). Electroejaculation has been shown to produce acute stress in cats, as indicated by changes in cortisol levels, but similar stress responses are seen in nonstimulated cats during recovery from anesthesia (Carter et al., 1984). Electroejaculation also may result in considerable retrograde ejaculation, with 6-100% (mean 59%) of the total ejaculated sperm cells recovered from the urinary bladder in one study (Herron et al., 1986). Domestic cats have been electroejaculated multiple times (>20) over a 32 week period without adversely affecting semen recovery (Pineda et al., 1984).

Recovery of sperm cells from the vas deferens after castration or death offers a means to salvage the genetic potential of valuable felids (Goodrowe and Hay, 1993). In nondomestic felids, this method usually is used for healthy animals that have died suddenly and unexpectedly, since prolonged illness frequently is associated with greatly diminished sperm cell production and quality. Sperm cells recovered from the epididymis are capable of fertilizing ova *in vitro* (Bowen, 1977; Niwa et al., 1985; Goodrowe and Hay, 1993) so salvaged spermatozoa might be cryopreserved and stored for future use (Howard et al.,

1986). However, while ejaculated domestic and nondomestic cat sperm cells have proven capable of post-thaw fertilization (Platz et al., 1978; Howard and Doherty, 1991; Donoghue et al., 1992a), similar functionality for post-thaw epididymal cat spermatozoa has not been demonstrated.

Exogenous Gonadotropins

Physiochemical Structure

As part of protocols for artificial insemination or *in vitro* fertilization, the stimulation of ovarian follicular development and/or final follicular maturation or ovulation are of critical importance. The ability to manipulate ovarian function in cats is the cornerstone for the application of assisted reproductive procedures in felids. Over the years, several stimulation regimens, using various combinations of exogenous gonadotropins, have been developed for domestic cats, depending on the desired outcome of the procedure. These regimens typically use either equine chorionic gonadotropin (eCG; also known as pregnant mare serum gonadotropin, PMSG) (Goodrowe et al., 1988a; Johnston et al., 1991a; Howard et al., 1992a), porcine or human follicle stimulating hormone (pFSH; hFSH) (Dresser et al., 1987; Goodrowe et al., 1988b; Orosz et al., 1992) or human menopausal gonadotropin (hMG) (Orosz et al., 1992) to promote development of ovarian follicles and either human chorionic gonadotropin (hCG) (Dresser et al., 1987; Goodrowe et al., 1988a; Johnston et al., 1991a; Howard et al., 1992; Orosz et al., 1992), human luteinizing hormone (hLH) (Orosz et al., 1992) or a synthetic peptide, gonadotropin releasing hormone (GnRH) (Chakraborty et al., 1979; Goodrowe and Wildt, 1987) to induce final maturation and/or ovulation of these follicles. Each of these gonadotropins have particular beneficial and detrimental effects, which are discussed in the following sections.

All of the gonadotropins (eCG; hCG; LH; FSH) are glycoproteins, with very similar basic structures, and consist of noncovalently linked alpha and beta chains (Birken and Canfield, 1978; Pierce and Parsons, 1981). The alpha chains have been shown to be

markedly conserved among species (~80% homology) and generate little biological activity separately from the beta chain (Papkoff, 1974; Farmer and Papkoff, 1979; Christakos and Bahl, 1979). The beta chain confers the specificity to the gonadotropins, probably through the nonhomologous (15-30%) portion of the proteins (Pierce, 1971; Moore et al., 1979). This nonhomologous fraction, however, probably also is responsible for the immunological responses when injected into a foreign host (Birken and Canfield, 1978; Farmer and Papkoff, 1979; Pierce and Parsons, 1981). While these gonadotropins have similar structure, they differ greatly in molecular weight, primarily due to variable amounts of carbohydrate attached to the peptide chains. The molecular weight of eCG is reported at ~65 kd (Christakos and Bahl, 1979) while hCG is ~37 kd (Birken and Canfield, 1978) and LH and FSH are both ~26-30 kd (Pierce and Parsons, 1981). This carbohydrate component consists of a high percentage of sialic acid and various oligosaccharides (galactose, glucosamine, galactosamine) (Moore et al., 1979; Pierce and Parsons, 1981). Desialylation markedly alters the biological activity of the gonadotropin, however, this effect is not due to alterations of the active site on the protein but rather to reduction in the circulatory persistence of the gonadotropins (Kalyan et al., 1982; Aggarwal and Papkoff, 1985; Martinuk et al., 1991). Presumably, the carbohydrate component prolongs the circulatory half-life of gonadotropins, a theory supported by pharmacokinetics data in a number of species.

The excretory half-life of eCG (~45% carbohydrate composition) (Moore et al., 1979) has been reported at 21 hours in sheep (McIntosh et al., 1975) to 51 hours in cattle (Menzer and Schams, 1979) while hCG, with just a ~30% carbohydrate composition (Pierce and Parsons, 1981), has an excretory half-life ranging from 48 minutes in rats (Kalyan et al., 1982) to ~24 hours in primates (Stouffer et al., 1986). By comparison, LH and FSH (with ~16% carbohydrate) (Pierce and Parsons, 1981) have half-lifes in rats of 20 minute and 110 minutes, respectively (Gay et al., 1970) and LH has an estimated half-life in cats of 15 minutes to 1 hour (Concannon et al., 1989). In contrast to the gonadotropins,

GnRH is a simple decapeptide (MW ~1.5 kd) with a circulatory persistence in cats of just a few minutes, based on LH secretory responses of cats injected with GnRH (Chakraborty et al., 1979; Concannon et al., 1989).

Biological and Immunological Properties

The biological properties of exogenous gonadotropins presumably depends on binding at endogenous gonadotropin receptors in the ovaries to induce "natural" physiological responses. Studies have shown that eCG may have both FSH-like and LH-like activity (Licht et al., 1979; Moore et al., 1979), possibly due to the presence of a unique determinant loop on the β-subunit (Ward and Moore, 1978). The primary biological action of eCG may be to rescue preantral follicles from atresia and thus increase the pool of potentially gonadotropin-responsive follicles (Monniaux et al., 1984; Boland et al., 1991). In contrast, hCG exhibits primarily LH-like activity (Birken and Canfield, 1978; Pierce and Parsons, 1981) although some intrinsic FSH-like activity has been detected (Louvet et al., 1976; Goodrowe and Wildt, 1987). Given the extreme homology of FSH and LH between mammalian species (Pierce and Parsons, 1981), exogenous FSH and LH are assumed to behave biologically like the native proteins to induce follicular growth and final follicular maturation/ovulation, respectively.

The immunological properties of the gonadotropins are dictated, to a large extent, by their secondary and three dimensional structure. While gonadotropins typically have a low content of α-helix (5-8%), a high percentage (25-71%) of β-structures (β-turns and β-sheets) are present (Pierce and Parsons, 1981). These secondary and tertiary structures frequently represent hydrophilic portions of the molecule and, as such, are readily accessible to the immune system of a foreign host.

Numerous studies in various mammalian species have indicated that the repeated administration of exogenous gonadotropins, singly or in combination, may result in decreased ovarian responsiveness, a probable immunologically-mediated consequence. Multiple treatments (at variable intervals) with hCG alone has been shown to cause

refractoriness to repeated induction of ovulation with hCG in mice (Land and McLaren, 1967) and rabbits (Greenwald, 1970; Reel et al., 1976), and to attenuate the responsiveness of primates to hCG (Ottobre and Stouffer, 1985). Similarly, single (Jainudeen et al., 1966; Bavister et al., 1986) or multiple (Alwan et al., 1988) treatments with eCG alone results in substantial reductions in ovarian responses (number of follicles or CL) on subsequent eCG treatments in cattle (Jainudeen et al., 1966; Alwan et al., 1988) or primates (Bavister et al., 1986). Combination regimens of eCG and hCG or, in one study (Maurer et al., 1968), pFSH and pLH generate a similar ovarian refractoriness after multiple treatments in mice (Lin and Bailey, 1965), cattle (Willett et al., 1953) and rabbits (Adams, 1953; Maurer et al., 1968).

This refractoriness to repeated stimulation is reportedly mediated through the humoral immune response directed against these exogenous gonadotropins. This contention is supported by the results of bioassays using sera from refractory animals to neutralize exogenous gonadotropins *in vivo* (Maurer et al., 1968; Reel et al., 1976; Jainudeen et al., 1966), by *in vitro* assays demonstrating precipitating antibodies or hemagglutination inhibition (Greenwald, 1970) and by radioimmunoassay (Reel et al., 1976; Ottobre and Stouffer, 1985) and ELISA (Bavister et al., 1986) data indicating anti-gonadotropin immunoglobulin titer levels. Interestingly, the presence of immunoglobulins directed against the exogenous gonadotropins does not apparently interfere with natural reproductive function. In refractory primates (Bavister et al., 1986), mice (Lin and Bailey, 1965; Land and McLaren, 1967), cattle (Jainudeen et al., 1966) and rabbits (Greenwald, 1970; Reel et al., 1976), females retained the capacity to cycle, ovulate and/or bear offspring in response to their endogenous gonadotropins.

The use of alternative gonadotropin regimens may be one effective method for overcoming immune-mediated ovarian refractoriness to a specific combination of exogenous gonadotropins. For example, rabbits that are refractory to injections of eCG and hCG for superovulation exhibit a rebound in ovarian responsiveness following

treatment with a regimen of pFSH and pLH (Maurer et al., 1968). As another example, rabbits that are anovulatory following repeated administration of hCG will ovulate in response to treatment with GnRH (Reel et al., 1976). However, this approach may not be practical with all species, such as in certain primates. In rhesus monkeys, immunoglobulins formed against eCG exhibit cross-reactivity with pFSH, presumably precluding the use of this alternative gonadotropin for ovarian stimulation (Bavister et al., 1986). Another strategy might be to limit the potential immunological consequences initially by avoiding the use of exogenous gonadotropins whenever possible. For example, in contrast to exogenous gonadotropins, repeated injection of rabbits with GnRH for ovulation induction did not cause the development of ovarian refractoriness, even after as many as 18 successive treatments at 3-week intervals (Reel et al., 1976). While considerable research has been conducted in other species, the effects of repeated treatment with exogenous gonadotropins on ovarian responsiveness and the potential immunological consequences have never been investigated in the domestic cat.

Beyond immunological considerations, the nature of some exogenous gonadotropins and their persistence in circulation are of additional concern in relation to their potential effect upon the maternal environment. In particular, the use of eCG for ovarian stimulation has been implicated as one factor that may contribute to poor embryo quality, especially in cattle (Bouters, 1983; Booth et al., 1985). The prolonged circulatory persistence of eCG is thought to promote the development of ancillary follicles post-ovulation in cattle and to increase levels of estradiol at an inopportune time (Saumande, 1980; Booth et al., 1985). Similarly, cats treated with eCG/hCG regimens for IVF or AI procedures have been reported to exhibit numerous ancillary follicles or secondary CL at 7 or 8 days post-hCG treatment (Goodrowe et al., 1988a; Donoghue et al., 1992b; Howard et al., 1992a). Studies in humans (Gidley-Baird et al., 1986) and mice (Gidley-Baird et al., 1986; Safro et al., 1990) have indicated that elevated estradiol concentrations and

altered estradiol to progesterone ratios may adversely affect embryo metabolism, embryo survival and implantation rates.

In cattle, numerous studies have investigated the administration of anti-eCG immunoglobulins to potentially neutralize residual eCG and avoid ancillary follicle formation (Dhondt et al., 1978; Kummer et al., 1980; Dieleman et al, 1989; Zeitoun et al., 1991; Bevers et al., 1993; Dieleman et al., 1993). In many earlier studies, the results were equivocal because the administration of anti-eCG immunoglobulins was poorly timed (Dieleman et al., 1993). Recent evidence suggests that anti-gonadotropin treatment must be initiated shortly after the endogenous LH surge to be effective (Dieleman and Bevers, 1987). Anti-eCG treatment reduces eCG levels to nondetectable levels within 2 hours of injection (Dieleman and Bevers, 1987) and, with proper timing (6-18 hours post-LH peak), has been shown to improve ovulation rate (Dieleman and Bevers, 1987), increase the number of quality embryos (Dieleman et al., 1989; Bevers et al., 1993) and reduce the number of secondary follicles and luteinized cysts (Dieleman et al., 1989; Bevers et al., 1993). Limited studies in mice (Katagiri et al., 1991) and silver foxes (Douglas et al., 1993) also have examined the effect of anti-eCG immunoglobulins on the persistence and residual activity of eCG, but similar studies have not been reported for the cat.

Ovulation Induction

Exogenous gonadotropin regimens may be used in cats to either induce ovulation in naturally-estrous females (Wildt and Seager, 1978a; Chakraborty et al., 1979), to induce follicular development with or without ovulation for natural breeding (Colby, 1970; Wildt et al., 1978b; Dresser et al., 1987) or artificial insemination (Platz et al., 1978; Howard et al., 1992a), and to promote follicular growth and maturation for IVF purposes (Bowen et al., 1977; Goodrowe et al, 1988a; Orosz et al., 1992; Pope et al., 1993a). Cats generally are considered to be induced ovulators (Greulich, 1934, Dawson and Friedgood, 1940), so that, in the absence of coital contact, queens in natural estrus require either vaginal stimulation with a glass rod (Greulich, 1934, Dawson and Friedgood, 1940) or treatment

with either human pregnancy urine (Foster and Hisaw, 1935; Windle, 1939), hCG (Hamner et al., 1970; Sojka et al., 1970; Wildt and Seager, 1978) or GnRH (Chakraborty et al., 1979) to cause ovulation of ovarian follicles. A single intramuscular injection of 250 or 500 IU hCG usually is sufficient to induce ovulation of most mature ovarian follicles but, to ensure ovulation in all estrual queens, two injections of hCG (100, 250, or 500 IU), administered on successive days, are required (Wildt and Seager, 1978). Similarly, a single intramuscular injection of GnRH, at a dosage level of 25 μg, will induce ovulation in most estrual queens (Chakraborty et al., 1979), but, as with hCG, two injections (at a 24-hour interval) are recommended (Schmidt, 1986).

A few studies have attempted to induce ovulation of nondomestic felids when in natural estrus. Most nondomestic felids are considered to be induced ovulators, although definitive proof is lacking for most species (Wildt et al., 1993). Based on laparoscopic findings and endocrine profiles, the tiger, jaguar and puma (Wildt et al., 1979b; Bonney et al., 1981; Seal et al., 1985) are probably induced ovulators while the lion, leopard, clouded leopard and cheetah (Schmidt et al., 1979; Schmidt et al., 1988; Wildt et al., 1993) may spontaneously ovulate, at least on occasion. In conjunction with AI procedures, ovulation induction in estrual nondomestic cats has been reported for the leopard (Dresser et al., 1982) and the lion (Bowen et al., 1982), using high dosages of hCG (1000-2000 IU) (Dresser et al., 1982; Bowen et al., 1982) and/or GnRH (100-200 μg) (Bowen et al., 1982).

Estrus Induction and Natural Mating

To induce follicular development and estrous behavior for natural mating purposes, multiple injections of eCG or pFSH have been given to anestrual queens (Colby, 1970; Wildt et al., 1978b; Cline et al., 1980; Dresser et al., 1987). Following multiple daily injections of eCG, some queens exhibited behavioral estrus, mated and conceived but, in general, this treatment regimen was associated with ovarian hyperstimulation, prolonged gestation lengths, a large number of unovulated follicles and poor neonatal survival (Colby,

1970; Wildt et al., 1978b; Cline et al., 1980). In contrast, treatment of queens with a single injection of eCG (100 IU) followed, 7 days later (at the time of mating), by hCG (50 IU) allowed for a high pregnancy rate (78%) and the production of normal-sized (~4.5 kittens) litters of healthy kittens (Cline et al., 1980).

Daily treatment of queens with pFSH (1-3 mg/day for 5-7 days) also may result in ovarian hypersensitivity and a large number of anovulatory follicles but, following natural mating, a satisfactory pregnancy rate (~70%) and litter size (5.8 kittens) may be obtained (Wildt et al., 1978b). However, in another study, queens treated with a similar daily pFSH regimen and naturally mated were reported to experience a high percentage of mid-gestational abortions, presumably due to luteal insufficiency (Tsutsui et al., 1989b). In addition, pFSH-stimulated/mated queens exhibited more unfertilized oocytes and fewer quality morula and blastocysts compared to queens bred in a natural estrus. Estradiol concentrations also were lower and progesterone levels rose earlier than in the natural estrus queens, indicating that this pFSH regimen probably is not optimal for assisted reproduction in cats.

Alternative pFSH regimens, using daily FSH injections but at lower dosages, have been investigated in domestic cats (Dresser et al., 1987). Queens were injected daily for the first 6 days with pFSH (4 mg total dosage) in conjunction with hCG (375 IU) treatment on the sixth and seventh day, and then naturally mated. While flushing the reproductive tracts allowed the recovery of a high number of quality embryos (~16/female), queens still exhibited multiple unovulated follicles (~10/female), and the capacity of queens to carry the pregnancies to term was not examined. Another study (Verstegen et al., 1993b) investigated the effects of an ultra-pure formulation of pFSH on ovarian stimulation, using a similar injection and mating schedule to Dresser et al. (1987). However, queens treated with the two higher FSH dosages (total dosage of 100 or 300 μg) had excessive numbers of unovulated follicles and degenerate embryos while queens receiving the lowest dosage (25 μg) had fewer unovulated follicles but very few recovered embryos.

Artificial Insemination

Similar ovulation stimulation regimens have been used as components of AI protocols in domestic and nondomestic cats. Sojka et al. (1970) reported the first successful AI in the domestic cat, by treating estrual queens with hCG (50 IU) to induce ovulation and performing intravaginal insemination (and also vaginal stimulation) of the unanesthetized female. Queens inseminated once with 5 - 50 $x10^6$ fresh sperm cells at the time of hCG injection had a pregnancy rate of 50%, but a second insemination 24 hours later increased this percentage to 75%. In a follow-up study, Platz et al. (1978) proved that AI pregnancies were possible in queens stimulated with a combination pFSH/hCG regimen and that frozen-thawed cat spermatozoa were capable of fertilization *in vivo*. Although pregnancy rates were low (~11%), this study was the first to demonstrate the potential of sperm cell cryopreservation, in association with AI, for use in the conservation of nondomestic felids.

Recently, combination regimens of eCG and hCG have been used successfully in conjunction with laparoscopic AI procedures in domestic cats (Howard et al., 1992a). With this procedure, queens are injected with eCG (100 IU), followed in 80 hours with hCG (75 IU), and then inseminated via laparoscopy 35-38 hours later. Pregnancy rates are reportedly ~50% but litter sizes are relatively small (~2 kittens/litter). However, this technique has been extrapolated to nondomestic felids and viable offspring have been generated by laparoscopic AI in the cheetah (Howard et al., 1992b), tiger (Donoghue et al., 1993a), puma (Barone et al., 1994), clouded leopard (Howard et al., 1993a), leopard cat (Howard and Doherty, 1991) and ocelot (Swanson, unpublished). Earlier studies have reported AI by vaginal insemination in gonadotropin-stimulated nondomestic felids (Phillips, 1981; Moore et al., 1981; Reed et al., 1981; Bowen et al., 1982; Dresser et al., 1982) but few of these attempts were successful, presumably due to poor sperm cell transport in anesthetized cats and anesthetic interference with the ovulatory process (Howard et al., 1992a).

In Vitro Fertilization

Combination regimens of exogenous gonadotropins also have been employed extensively with protocols for IVF. In the initial study of domestic cat IVF, oocytes were obtained from the oviducts of queens stimulated with eCG (150 IU) and induced to ovulate with hCG (50 IU) given 72 hours later. Recovered oocytes were inseminated with sperm cells capacitated *in utero* and 62% of the oocytes were fertilized (Hamner et al., 1970). Bowen (1977) demonstrated that *in utero* capacitation was not required by inseminating oocytes, recovered from the oviducts of eCG/LH stimulated queens, with spermatozoa obtained from the ductus deferens. Fertilization rate was reported at 78-81% in two different fertilization media with initial embryo cleavage observed 20-28 hours post-insemination. Niwa et al. (1985) stimulated queens with eCG (150 IU), followed by hCG (100 IU) 72 hours later, and recovered oocytes from the oviducts 31-32 hours post-hCG. Oocytes were inseminated with epididymal spermatozoa in a modified Kreb's medium (mKrb; Toyoda and Chang, 1974) and sperm were observed to penetrate the zona pellucida and form pronuclei within 4 hours of insemination. The ovarian stimulation and fertilization protocols for this study (Niwa et al., 1985) were slightly modified in a subsequent study by Goodrowe et al (1988a), who compared the effect of hCG dosage (100 vs. 200 IU) and the timing of hCG injection relative to eCG (72 vs. 80 hours) on IVF success.

In contrast to previous IVF studies, oocytes for this study were recovered from pre-ovulatory ovarian follicles by transabdominal laparoscopic aspiration and sperm cells for insemination were collected by electroejaculation. The results indicated that the lower hCG dosage (100 IU) produced fewer degenerate oocytes and that the longer eCG-hCG interval (80 hours), in combination with the low hCG dosage, resulted in the highest fertilization (48%) and cleavage (45%) rates. In addition, the results demonstrated that electroejaculated spermatozoa, subjected to swim-up processing (Howard et al., 1990), were capable of fertilization without *in utero* exposure and that aspirated follicles form

functional CL with relatively normal secretory profiles. Finally, this study demonstrated that IVF embryos are biologically competent, by producing multiple offspring following embryo transfer. A more extensive study of eCG-hCG intervals (Donoghue et al., 1992b) determined that, compared with longer intervals, an 80- or 84-hour interval between eCG and hCG injections actually produced the greatest number of quality oocytes (17-18/female) and the greatest number of quality embryos (10-11/female) post-insemination and generated the highest circulating estradiol levels. The results of this study indicated that prolonging the interval excessively between the FSH-like and LH-like signals may compromise intrafollicular oocyte maturation.

Using an 84-hour interval between eCG and hCG for ovarian stimulation, two follow-up studies (Johnston et al., 1991a,b) examined the effect of media complexity, protein source, temperature and gas atmosphere on fertilization (and embryo culture). Similar fertilization rates (70-80%) were obtained following insemination in two simple media, mKrb and a modified Tyrode's solution (TALP) (Bavister and Yanagamachi, 1977), and in a complex medium, Ham's F-10 (HF-10) (Ham, 1963), but with bovine serum albumin (BSA; 0.4%) as the protein source for all media. However, when HF-10 was supplemented with one of four different protein sources (polyvinylalcohol, PVA; BSA; fetal bovine serum, FBS; estrual cat serum, ECS), fertilization rates were greater in HF-10 with 5% FBS (84%) or 5% ECS (85%) than in HF-10 with PVA (67.3%) but with HF-10 with BSA (76%) intermediate to the other media. These results suggested that the type of media and protein supplement had minimal effect on the success of *in vitro* fertilization in the cat. The second study (Johnston et al., 1991b) compared *in vitro* fertilization rates (and embryo culture) at three different temperatures (37°C; 38°C; 39°C) and in three different gas atmospheres (5% CO_2 in air; 10% CO_2 in air; 5% CO_2, 5% O_2 and 90% N_2) and showed that these variables had little effect of fertilization success (~75% in all treatments).

In a more recent study (Roth et al., 1994a), the influence of oviductal cell co-culture on fertilization rates was examined and similar levels of IVF were observed with insemination on cellular monolayers (65% fertilization) and in medium (HF-10) alone (59%). One final study (Donoghue et al., 1993b) compared the IVF capability for oocytes obtained from eCG/hCG-stimulated queens to that for oocytes obtained from queens on the initial days of natural estrus. Follicular maturation was induced in queens on the first, second and third day of natural estrus with injections of hCG and recovered oocytes were fertilized *in vitro*. Queens treated with hCG on the first day of estrus displayed fewer follicles and more degenerate or immature oocytes than eCG/hCG treated queens or queens treated with hCG on the second or third day of estrus, indicating that sexual receptivity may be asynchronous with follicular maturity for an LH-like (mating) stimulus. In addition, this study suggested that oocytes and early-stage embryos resulting following eCG/hCG treatment may not be compromised relative to those obtained from queens in natural estrus.

Several alternative exogenous gonadotropin protocols have been evaluated for their usefulness with IVF. Pope et al. (1989a) treated queens with a pFSH/hCG regimen similar to previously described regimens used with natural breeding (Dresser et al., 1987). Queens were injected with daily, decreasing dosages of pFSH (2-4 mg total dosage) for 4 days and then administered hCG (100 IU) on the fifth day. Oocytes were aspirated at laparotomy 25-27 hours post-hCG and inseminated with spermatozoa (collected by AV) in Ham's F-10 medium (with 5% FBS). The number (~26 oocytes/female) and quality (87% mature) of recovered oocytes and the fertilization rate (~64%) were comparable to values reported with eCG/hCG treatment (Donoghue et al., 1992b).

Another study (Peterson et al., 1991) compared an pFSH infusion pump to daily FSH injections for ovarian stimulation. Queens received continuous infusion of FSH (1.2, 2.4 or 4 mg total dosage) over a 5 day period from a subcutaneous mini-osmotic pump, followed by hCG (375 IU) at the time of pump removal. Queens administered FSH via the pump tended to have more follicles and mature oocytes than queens injected once daily with

FSH (4 mg total dosage) but fertilization percentages were not reported. Lastly, regimens of hFSH/hCG and hMG/hCG have been evaluated in domestic cats (Orosz et al., 1992) with the results indicating that both regimens were capable of inducing follicular development (17-21 oocytes/female) but with low fertilization rates (19-26%), and suggesting that an LH component was required in conjunction with the FSH-like stimuli to promote follicular estradiol synthesis.

Both eCG/hCG and pFSH/hCG regimens have been used for IVF of nondomestic cat species, extrapolating from findings with domestic cats. Using similar protocols but, in some cases, slightly higher eCG/hCG dosages, procedures for ovarian stimulation, laparoscopic follicular aspiration and IVF have been investigated in the tiger (Donoghue et al., 1990), cheetah (Donoghue et al., 1992c), puma (Miller et al., 1990) and leopard cat (Goodrowe et al., 1989b). Ovarian responsiveness and oocyte quality varied by species but oocyte fertilization (26-70% of oocytes) and embryo development was observed with each species, showing the potential of this technology for felid conservation. The biological competence of IVF-derived embryos from the tiger has been demonstrated following embryo transfer and the birth of offspring (Donoghue et al., 1990). Regimens of pFSH/hCG have been used with the smaller felids, specifically the Indian desert cat, jungle cat, fishing cat and black-footed cat (Pope et al., 1989a; Pope et al., 1993a). Mature oocytes were obtained from all species but the number of mature oocytes and/or the fertilization rates were low except for the Indian desert cat. In the latter species, a moderate number (~11/female) of mature oocytes were recovered at laparotomy and ~63% of these oocytes fertilized. These IVF-derived embryos proved developmentally competent following embryo transfer and the birth of one viable kitten (Pope et al., 1989b).

Embryo Culture

Early studies of IVF in cats (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985; Goodrowe et al., 1988a) were more concerned with events associated with sperm cell capacitation, oocyte penetration and initial embryo cleavage than with the culture

requirements of IVF-derived embryos. Hamner et al. (1970) reported embryo development to the 16-cell stage after three days of culture in rabbit serum while Bowen (1977) observed cleavage of IVF embryos past the 8-cell stage when cultured in modified HF-10 (with BSA) with three embryos forming blastocysts. Finally, Goodrowe et al. (1988a) described the development of IVF embryos to the 16-cell stage after 72-96 hours of culture in mKrb medium. The first comprehensive studies of cat embryo development was conducted by Johnston et al. (1991a,b), who examined the effects of media complexity, protein source, temperature and gas atmosphere on IVF and embryo cleavage. In the first study, IVF-derived embryos were shown to develop readily to the morula stage (78-95% of embryos) but with greater morula formation when cultured in HF-10 (with BSA) than in TALP (with BSA), indicating that a more complex media (containing supplemented amino acids, vitamins and minerals) probably was preferable to a more simple media. Comparison of the effect of protein sources (PVA; BSA; FBS; ECS) in a complex medium (HF-10) on embryo development revealed that embryos formed morulae equally well in all protein sources (83-98% of embryos) but that FBS and ECS were preferable to BSA and PVA for the formation of blastocysts. However, even in the presence of FBS and ECS, only 22-30% of morulae became blastocysts, indicating a pronounced morula-to-blastocyst developmental block in this species. In the second study (Johnston et al., 1991b), variations in temperature and gas atmosphere had no effect on embryo development to the morula stage or on the ability to circumvent the developmental block. The persistence of this block, despite multiple alterations in culture conditions, brings into question the developmental competence of the majority (~70%) of the domestic cat embryos generated by exogenous gonadotropin treatment and IVF.

The developmental arrest *in vitro* at the morula stage in cats is similar in timing to the late stage block reported in the rabbit (Kane and Foote, 1970), but the addition of amino acids to the culture medium resolved this problem in the latter species. Embryos from most other species exhibit developmental blocks much earlier in culture, specifically at the 2-cell

stage (mouse, Cross and Brinster, 1973; hamster, Schini and Bavister, 1988), 4-cell stage (pig, Davis and Day, 1978; hamsters, Schini and Bavister, 1988) and 8- to 16-cell stage (cattle, Thibault, 1966; hamster, Bavister et al., 1983; sheep, Gandolfi and Moor, 1987). In many of these species, these early blocks are thought to be associated with the maternal-to-zygotic transition in genomic control, a period in which the embryo initiates its own mRNA and protein synthesis (Frei et al., 1989; Telford et al., 1990). In the domestic cat, the timing of the maternal-to-zygotic transition has not been firmly established, but it is unlikely to occur as late as the morula stage, based on evidence from other mammalian species.

In many species, *in vitro* developmental blocks have responded to alterations in culture conditions, specifically changes in basic energy sources and/or amino acids, and co-culture of embryos with various cell types. In mice, the substitution of glutamine for glucose in the culture medium during the first 48 hours of culture allows embryos to circumvent the 2-cell block; however, glucose was required after this period for maximal blastocyst formation (Chatot et al., 1989). Another study suggested that early stage mice embryos preferentially use glutamine as an energy source (Chatot et al., 1990). Similarly, in hamsters, the removal of glucose and/or inorganic phosphate from the culture medium permitted a high percentage of hamster embryos to bypass the 2-cell, 4-cell and 8-cell blocks (Schini and Bavister, 1988; Seshagiri and Bavister, 1989). In these studies, the presence of glucose and phosphate in the culture medium was thought to create a "Crabtree Effect", in which these substances enhance glycolysis and subsequently inhibit oxidative metabolism (Seshagiri and Bavister, 1991).

In contrast, the presence of glucose during early culture (in conjunction with tissue co-culture) of cattle embryos apparently does not adversely affect development through the block to the morula stage, but decreases the percentage of embryos that are capable of forming blastocysts (Ellington et al., 1990). This implies that early alterations in energy pathways by glucose may have detrimental effects not seen until much later in culture. The

addition of various amino acids, other than just glutamine, also have proven beneficial in overcoming developmental blocks in some species, such as the rabbit (Carney and Foote, 1970) and the hamster (Carney and Bavister, 1987). Collectively, these studies give some indication that the energy substrates required by embryos are not static during *in vitro* culture. For example, in mice, improved development of embryos was obtained by altering the levels of pyruvate, lactate and/or glucose throughout embryo culture (Brown and Whittingham, 1992), implying that preferred energy substrates change with stage of embryo development (Brison and Leese, 1991; Rieger et al., 1992; Rieger, 1992). The effects of different energy sources and their temporal availability have not been reported for embryo development in the cat.

Co-culture of embryos with various cell types (primarily cumulus, oviduct and uterine cells) or with medium conditioned on these cells has allowed circumvention of the developmental block and improved blastocyst formation in a number of species, including the cow (Eyestone and First, 1989; Ellington et al., 1990; Eyestone et al., 1991; Zhang et al., 1992), sheep (Gandolfi and Moor, 1987), pig (White et al., 1989), goat (Sakkas et al., 1989; Prichard et al., 1992) and rabbit (Carney and Foote, 1990). In several species, studies have suggested that oviduct cells are superior to other cell types and/or conditioned medium for embryo culture (Gandolfi and Moor, 1987; Rexroad and Powell, 1988; Ellington et al., 1990). The precise mechanism by which oviductal cell co-culture benefits embryo development is unknown, but it is postulated to either contribute embryotrophic factors to the medium or to remove embryo-suppressive factors from the culture environment (Eyestone et al., 1991).

Recently, co-culture of IVF-derived cat embryos with oviductal cell monolayers was investigated in an attempt to overcome the morula developmental block (Roth et al., 1994a). Monolayers were established using frozen-thawed, subpassed cells from a defined oviductal epithelial cell line and IVF-derived embryos were cultured on the monolayers in HF-10 (with 10% FBS). Embryo development to the morula stage was similar with co-

culture (71% of embryos) and in medium alone (78% of embryos) but no blastocysts were formed with either treatment. The failure of oviductal cell co-culture to promote blastocyst formation in cats suggests that the nature of the block is somewhat unique compared with other species, but variations of cell preparations and culture conditions need to be investigated before co-culture can be dismissed entirely. However, these results do little to improve confidence in the biological competence of most IVF-derived cat embryos.

Another recent study (Roth et al., 1994b) has compared the *in vitro* development of IVF-derived embryos and embryos generated *in vivo* by natural mating. This study indicated that IVF and *in vivo*-produced embryos develop at a similar rate during *in vitro* culture but, while IVF-derived embryos arrest at the morula stage, *in vivo*-produced embryos (71%) develop readily to the blastocyst stage. This finding suggests that IVF-derived cat embryos may have an intrinsic defect in developmental capacity. However, because the duration of culture had a negative effect on blastocyst formation by *in vivo*-produced embryos, culture conditions also can be considered suboptimal. The results of this study suggest that the true competence of IVF-derived embryos probably can only be assessed by transferring embryos to suitable recipients and evaluating development through both the morula block stage and to parturition.

Limited studies of IVF-derived embryo development *in vitro* have been conducted in a few nondomestic felid species, including the tiger (Donoghue et al., 1990), cheetah (Donoghue et al., 1992c) and Indian desert cat (Pope et al., 1989a). Following eCG/hCG stimulation and IVF, tiger embryos developed to the morula (44%) or early blastocyst (30%) stage within 96 hours of culture in HF-10, while cheetah embryos typically developed to the early morula stage (53%) after 72 hours of culture. In the Indian desert cat, following pFSH/hCG stimulation and IVF, most (63%) embryos developed to the morula stage by 96 hour of *in vitro* culture. These studies suggest that IVF-derived embryo developmental requirements are similar (at least to the morula stage) between

nondomestic species and domestic cats, and indicate that domestic cats may be an appropriate felid model for studying embryo developmental factors in general.

Embryo Transfer

The first successful embryo transfer in the cat was reported by Kraemer et al. (1979), using embryos generated by mating queens in natural estrus and flushing the reproductive tracts at laparotomy 6-9 days later. Recipients were synchronized by mating estrual queens with a vasectomized male or by two injections of hCG, and embryos (n=47) were surgically transferred to the uterine horns of nine recipient females. Although four queens became pregnant, only two litters and four total kittens were produced. Embryo transfer also has been performed successfully using embryos recovered from the uteri of naturally-mated queens in natural or pFSH-induced estrus.(Goodrowe et al., 1988b; Tsutsui et al., 1989b). In one study (Goodrowe et al., 1988b), embryos (n=~66) were transferred at laparotomy to the uterine horns of pFSH/hCG-synchronized recipients (n=15) and three pregnancies resulted with three kittens born. Similarly, in another study (Tsutsui et al., 1989b), embryos (n=38) recovered from the uteri of naturally-mated queens previously treated with pFSH were transferred surgically to the uteri of pFSH/hCG-synchronized recipients (n=6). All six recipient females were diagnosed by ultrasound as pregnant but five aborted prior to term (~days 20-40 of pregnancy) and one queen, treated with progesterone throughout pregnancy, had two kittens by cesarean section. The successful transfer of frozen-thawed cat embryos also has been reported (Dresser et al., 1988), using embryos originally flushed from pFSH/hCG-treated, naturally-mated queens with subsequent post-thaw transfer to pFSH/hCG-synchronized recipient females.

Embryos derived from IVF also have been successfully transferred on a number of occasions, proving the biological competence of at least some embryos generated in this manner. Goodrowe et al. (1988a) transferred 2- to 4-cell stage IVF-derived embryos (n=68) at laparotomy to the oviducts of eCG/hCG synchronized recipients (n=6), and produced five litters and a total of 10 kittens. Subsequently, Pope et al. (1989a) cultured

IVF-derived embryos, obtained from pFSH/hCG treated queens, to the morula stage and surgically transferred embryos (n=190) to pFSH/hCG-synchronized recipients (n=17), resulting in the birth of only five litters and a total of 12 kittens. Additional studies (Peterson et al., 1991; Orosz et al., 1992) have reported births following surgical transfer of IVF-derived embryos, but similar to the earlier studies, pregnancy and/or embryo survival rates were universally low.

At present, the transfer of IVF embryos to recipients synchronized with exogenous gonadotropins exhibits a very low level of reproductive efficiency. Even when pregnancy rates are fairly high (>50%) (Goodrowe et al, (1988a), embryonic and fetal mortality is usually severe (<10% embryo survival) and litter sizes are typically small (1-2 kittens). The potential reasons for the low reproductive efficiency with IVF/ET are multiple, but the two most probable causes are that 1) IVF embryos have a compromised biological competence and 2) the maternal environment of exogenous gonadotropin-treated recipients is unsuitable. These possibilities have been discussed in more detail in the preceding sections.

Once these other issues are resolved, another potential problem with IVF/ET in cats is that, to date, all transfers have required surgical (laparotomy or laparoscopy) approaches. In many other species, including man (Steptoe and Edwards, 1978), cattle (Rowe et al., 1980), horses (Allen and Rowson, 1975), primates (Pope et al., 1983), rabbits (Testart, 1969), rats (Vickery et al., 1969) and mice (Moler et al., 1979), embryo transfers have been performed using transcervical (i.e., nonsurgical) techniques. Transcervical embryo transfer avoids many of the potential complications of surgical procedures (wound dehiscence, increased opportunity for infection, hemorrhage, adhesions) and usually reduces the level of veterinary skill that is required. In cats, a technique for transcervical catheterization has been described for embryo recovery (Hurlbut et al., 1988) but transcervical embryo transfer has not been reported.

With nondomestic cat species, the transfer of IVF-derived embryos has resulted in births of viable offspring only in the tiger (Donoghue et al., 1990) and the Indian desert cat (Pope et al., 1989b). Donoghue et al. (1990) transferred 2- to 4-cell tiger embryos (n=82) to the oviducts of six eCG/hCG-treated recipient females. One recipient became pregnant and three cubs were subsequently delivered by cesarean section. Pope et al. (1989b) transferred Indian desert cat embryos (n=40) to pFSH/hCG-synchronized domestic (n=5) and Indian desert cat (n=1) recipient females. One domestic cat recipient was diagnosed as pregnant and gave birth to one viable and one stillborn Indian desert cat kitten. These two successes are encouraging, but, as with IVF/ET in the domestic cat, the efficacy of these techniques must be improved substantially to be of any significance in the conservation of endangered cat species.

CHAPTER II

PRODUCTION OF EXOGENOUS GONADOTROPIN-NEUTRALIZING IMMUNOGLOBULINS IN THE CAT AND RELEVANCE FOR ASSISTED REPRODUCTION IN FELIDS

Introduction

Exogenous gonadotropins, such as equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG), have been used for the stimulation of ovarian follicular development and/or final follicular maturation/ovulation in a number of mammalian species including cattle (Boland et al., 1991), rhesus monkeys (Wolf et al., 1990), rabbits (Maurer et al., 1968) and mice (Edwards and Fowler, 1960). Recently, as a component of successful *in vitro* fertilization (IVF) (Goodrowe et al., 1988a; Johnston et al., 1991a,b; Donoghue et al., 1992b; Swanson and Godke, 1994a) and artificial insemination (AI) (Howard et al., 1992a) strategies, combination regimens of eCG and hCG have been developed for use with domestic cats. These ovarian stimulation protocols also have been extrapolated to studies of IVF and AI in a number of nondomestic cat species, including the tiger (Donoghue et al., 1990; Donoghue et al., 1993a), puma (Miller et al., 1990; Barone et al., 1994), cheetah (Donoghue et al.1992c; Howard et al., 1992b) and leopard cat (Goodrowe et al., 1989b).

The continued development and refinement of these assisted reproductive techniques are critical for the propagation of genetically valuable domestic and nondomestic felid species (Wildt et al., 1990; Wildt et al., 1992b; Howard et al., 1993b). However, before these techniques are more widely applied to the reproductive management of felid populations, the potential immunological consequences of exogenous gonadotropin administration must be addressed. In several mammalian species, the injection of exogenous gonadotropins, either once (Bavister et al., 1986) or multiple times (Lin and Bailey, 1965; Jainudeen et al., 1966; Land and McLaren, 1967; Maurer et al., 1968; Greenwald, 1970; Reel et al., 1976; Ottobre and Stouffer, 1985), has been associated with a decrease in ovarian responsiveness on subsequent stimulation attempts. This ovarian

refractoriness to repeated ovarian stimulation is reportedly mediated through a humoral immune response (Jainudeen et al., 1966; Greenwald, 1970; Reel et al., 1976; Ottobre and Stouffer, 1985; Bavister et al., 1986), with gonadotropin-binding immunoglobulins attenuating the biological activity of these foreign proteins.

In our laboratory, a standard eCG/hCG regimen (Johnston et al., 1991a,b; Donoghue et al., 1992b) was used with domestic cats for the generation of IVF-derived embryos, but a pronounced decrease in ovarian responsiveness was observed when queens were stimulated repeatedly at short intervals (44-50 days). In the present study, a possible immunological mechanism for this ovarian refractoriness was investigated. The specific objectives of this study were to: 1) determine if eCG-binding immunoglobulins are present in sera of eCG/hCG-refractory queens, 2) examine the affinity of these immunoglobulins for other exogenous gonadotropins (hCG and porcine follicle stimulating hormone, pFSH), 3) assess the biological effect of these immunoglobulins *in vivo* using mouse ovarian stimulation assays and 4) evaluate the effectiveness of an alternative gonadotropin regimen for ovarian stimulation of eCG/hCG refractory queens.

Materials and Methods

Animals

Random-source, adult male (n=2) and female (n=16) domestic cats were obtained from a local animal control facility and conditioned for at least 3 weeks prior to experimental use. For initial health screening, cats were given a general physical examination and blood samples were collected to evaluate for possible feline leukemia virus infection (Feline Leukemia Virus Antigen Test Kit, Fermenta Animal Health, Omaha, NE). During conditioning, cats were housed in individual cages in a quarantine area and their general health status was assessed daily. Cats were vaccinated against feline rhinotracheitis, calici and panleukopenia viruses (Felocell VCR, Norden Laboratories, Lincoln, NE) and treated with praziquantel (Droncit, Mobay Corp., Animal Health

Division, Shawnee, KS) for possible tapeworm parasitism. At the end of the quarantine period, only clinically healthy animals were released for use in experimental studies. Following conditioning, cats were housed singly or in pairs in stainless steel cages (1 x 1 x 1 m) and maintained under a controlled artificial illumination cycle (12 hours light:12 hours dark). A commercial cat food diet (Science Diet, Hills Pet Products, Topeka, KS) and water were provided *ad libitum*.

Ovarian Stimulation

Gonadotropin-induced ovarian stimulation and laparoscopy followed previously established procedures (Wildt et al., 1977; Goodrowe et al., 1988a; Johnston et al., 1991a,b). Briefly, queens were monitored every 1-3 days for signs typical of behavioral estrus, such as lordosis, treading of the hind feet and increased vocalization (Michael, 1961). Anestrual queens were injected (i.m.) with 150 IU eCG (Sigma Chemical Company, St. Louis, MO) followed 84 hours later with an injection of 100 IU hCG (Sigma). At 24-27 hours post-hCG, queens were anesthetized with an i.m. injection (8 mg/kg body weight) of tiletamine-zolazepam hydrochloride (Telazol; A.H. Robbins Company, Richmond, VA), placed in dorsal recumbency and prepared for surgery. Queens were examined by laparoscopy (Wildt et al., 1977; Goodrowe et al., 1988a) to determine the number of mature vesicular follicles (≥2 mm in diameter) present on the ovaries. Mature follicles were transabdominally aspirated (Goodrowe et al., 1988a) to recover oocytes for use in IVF experiments and recovered oocytes were assessed for maturational status, with mature oocytes possessing a distinct corona radiata and expanded cumulus cell mass and immature oocytes having a tightly compacted cumulus cell investment (Goodrowe et al., 1988a; Johnston et al., 1991a,b).

Queens (n=8) receiving multiple eCG/hCG injections were stimulated at 44- to 50-day intervals for the first three procedures and at an 80- to 141-day interval for the fourth procedure. Six cats in this group were treated with eCG/hCG for a fifth stimulation procedure at a 102-day interval and then challenged 63 days later with a regimen of

pFSH/hCG. These queens were injected once daily (s.c.) with 1.0 mg pFSH (FSH-P; Schering-Plough Animal Health Corporation, Kenilworth, NJ) for 5 consecutive days, administered (i.m.) 100 IU hCG on the sixth day and subjected to laparoscopy 24-27 hours later for evaluation of ovarian response and for follicular aspiration. Blood was collected via jugular venipuncture from stimulated female cats (1x, n=6; 3x, n=8, 4x, n=8) at the time of laparoscopy, from male cats (n=2) and from naive female cats (n=8). Sera was stored frozen at -20°C until analyzed by ELISA or used in mouse ovarian stimulation assays.

Solid-phase ELISA

ELISA procedures were similar to that described by Bavister et al. (1986) with slight modification. eCG, hCG and pFSH (all obtained from Sigma) were diluted in 60 mM carbonate-bicarbonate buffer (pH 9.6) to a concentration of 20 ng protein/µl. Aliquots (25 µl) of the respective gonadotropin solutions were pipetted into wells (500 ng protein/well) of 96-well flat bottom microtiter plates (Immulon I, Dynatech Laboratories, Alexandria, VA) and the plates were incubated for 3 hours at 37°C. Plates were washed five times with 0.01M phosphate-buffered saline- 0.1% Tween 20 (PBS-Tw) and blotted dry. Thawed serum samples were diluted (1:100, 1:200, 1:400) with PBS-Tw and aliquots (100 µl) of each added to wells in triplicate. Plates were incubated for 1 hour at room temperature (22°C) and washed five times with PBS-Tw. Horseradish peroxidase (HRP)-conjugated, affinity purified rabbit anti-cat IgG (Zymed Laboratories, South San Francisco, CA) was added (100 µl ; 1:1000 dilution in PBS-Tw) to appropriate wells and incubated for 30 minutes at 37°C. The plates were washed and 100 µl of *o*-phenylenediamine solution (Sigma; 1 mg/ml 0.05 M citrate buffer, pH 4.5) with 0.05% H_2O_2 was added to each appropriate well. Microtiter plates were incubated in the dark for 40 minutes at 22 °C and 50 µl of 2.5 M H_2SO_4 was added to each well to stop the color reaction. Optical density (OD) of wells was measured at 492 nm using an automated microplate reader (MR5000 model, Dynatech).

Each microtiter plate included serum samples from each treatment group in addition to substrate, serum and enzyme conjugate controls. For assessment of exogenous gonadotropin binding for individual serum samples, mean OD values (± SD) were determined for the triplicate wells of each sample. Mean OD values above the mean value for the naive serum group +3 SD were considered positive (+) for the presence of gonadotropin-binding immunoglobulins.

Mouse Ovarian Stimulation Assay

Two separate mouse ovarian assays, using modified protocols described for other rodent species (Murphy et al., 1984; Bavister et al., 1986), were conducted to assess the pre-ovulatory and post-ovulatory effects of eCG-binding immunoglobulins *in vivo*. For each assay, mice (ICR strain; 25-27 days of age) were weighed and assigned to treatments to equalize mouse weights between groups. Sera with low (-sera; from naive cats) and high (+sera; from cats stimulated three times) eCG-binding immunoglobulin levels (as indicated by ELISA) were each pooled, mixed with equal volumes of eCG (100 IU/ml saline) and incubated for 15 minutes at 37°C. Mice (n=40, assay 1; n=48, assay 2) received intraperitoneal injections (100 µl) of either eCG (5 IU)/saline, eCG (5 IU)/-sera, eCG (5 IU)/+sera or saline alone. For the first or pre-ovulatory assay, mice were killed by cervical dislocation 48 hours following injection (i.p.) and the paired ovaries from each mouse were cleaned of extraneous tissue, blotted dry and weighed (± 0.01 mg). For the second or post-ovulatory assay, all mice received an injection (i.p.) of hCG (5 IU) 47 hours after the initial treatment and the reproductive tracts were harvested 20 hours later. Paired ovaries were weighed as in the first assay and the oviducts of each mouse were flushed with PBS (with 1% fetal bovine serum) to allow determination of the number of ovulated oocytes.

Statistical Analysis

The mean number (± SEM) of follicles (≥2 mm in diameter) and the proportion of recovered oocytes classified as mature were calculated for queens stimulated multiple times

with eCG/hCG and pFSH/hCG. Mean follicle number was evaluated using analysis of variance and differences between groups determined with a least significance difference (LSD) test (SAS, 1984). The proportion of mature oocytes for each stimulation period was compared using Chi square analysis (Steel and Torrie, 1960). For the mouse ovarian stimulation assays, the mean paired ovary weight (± SEM) and number of ovulated oocytes (± SEM) were calculated for each treatment group. Data were evaluated with analysis of variance and differences between groups were assessed with a LSD test.

Results

Repeated Ovarian Stimulation

Queens repeatedly stimulated with eCG/hCG at short intervals (44-50 days) demonstrated a significant decrease ($P<0.05$) in ovarian responsiveness (as measured by the number of mature follicles) by the third stimulation attempt (Table 1). A fourth series of eCG/hCG injections resulted in a further decrease ($P<0.05$) in ovarian follicular development, despite extending the stimulation interval from 44-50 days to 80-141 days. Queens (n=6) subjected to a fifth stimulation attempt at a 102-day interval exhibited a similar ($P>0.05$) mean number of mature follicles as observed with the third and fourth stimulation procedures. Oocyte maturation demonstrated a parallel decline to follicle number, with a lesser proportion ($P<0.05$) of oocytes recovered at the third, fourth and fifth stimulations exhibiting morphological characteristics typical of mature oocytes. Treatment of these same six queens 63 days later, however, using a pFSH/hCG regimen, resulted in a significant increase ($P<0.05$) in the mean number of follicles (9.5 ± 2.1) relative to the third, fourth and fifth stimulation attempts, but the proportion of mature oocytes (3/53, 5.7%) was not different ($P>0.05$) from that observed with the fourth and fifth stimulations.

Table 1. Number of ovarian follicles (≥2 mm) and proportion of recovered oocytes assessed as mature in queens following sequential treatments with eCG/hCG and pFSH/hCG

Animal number	Number of follicles observed with sequential eCG/hCG and pFSH/hCG treatments[a]					
	1st treatment	2nd treatment	3rd treatment	4th treatment	5th treatment	6th treatment
1	23	27	6	4	-	-
2	23	40	4	2	-	-
3	24	21	4	1	16	16
4	19	6	4	0	0	10
5	26	5	6	0	2	6
6	13	14	4	0	4	4
7	7	9	6	3	1	15
8	4	20	4	0	1	6
Mean number (± SEM) of follicles/queen	17.4 ± 3.0[b]	17.8 ± 4.2[b]	4.8 ± 0.4[c]	1.3 ± 0.6[d]	4.0 ± 2.5[cd]	9.5 ± 2.1[e]
Proportion (%) of recovered oocytes graded mature	70/84 (83.3%)[b]	75/86 (87.2%)[b]	6/29 (20.7%)[c]	3/10 (30.0%)[cd]	0/14 (0%)[cd]	3/53 (5.7%)[d]

[a] Queens were stimulated with eCG/hCG at 44- to 50-day intervals for the first three treatments, at 80- to 141-day intervals for the fourth treatment and at a 102-day interval for the fifth treatment. For the sixth treatment, queens were injected with pFSH/hCG after a 63-day interval. With each treatment, queens were subjected to laparoscopy 24-27 hours post-hCG and ovarian follicles were aspirated to recover oocytes.

[b-e] Values within rows with different superscripts are significantly different ($P<0.05$).

ELISA Assay

In the development of the ELISA for this study, optimal dilutions of serum samples (1:100, 1:200, 1:400) and HRP-conjugated anti-cat immunoglobulin (1:1000) were determined to produce OD values for most samples that were within the quantifiable range of the microplate reader (OD <2.0) and within the linear portion of the OD curve for serum serial dilutions. The concentration of eCG, hCG and pFSH in wells (500 ng) was chosen based on the optimal antigen concentration reported in a previous study (Bavister et al., 1986). Nonspecific binding was minimized by the use of a relatively high concentration (0.1%) of Tween 20 in PBS for sample and enzyme conjugate dilution and for plate washing. Blocking plates with 0.5% bovine serum albumin (BSA) prior to serum sample addition had no appreciable effect on OD values for any tested sample (data not shown) so this step was omitted.

Affinity of Immunoglobulins for eCG and other Exogenous Gonadotropins

ELISA data for eCG-binding indicated that sera obtained from naive queens (n=8) and diluted 1:100, 1:200, and 1:400 had mean OD values (± SD) of 0.199 ± 0.098, 0.118 ± 0.056 and 0.066 ± 0.029, respectively. Optical density values of individual serum samples above the naive sera means +3 SD (1:100, 0.493; 1:200, 0.286; 1:400, 0.153) were considered positive (+) for eCG-binding immunoglobulins. While none of the males (n=2), naive queens (n=8) or queens stimulated once with eCG/hCG (n=6) had positive (+) sera (Table 2), seven of eight queens stimulated three times and six of eight queens stimulated four times with eCG/hCG had positive (+) sera for eCG-binding (Table 3).

ELISA data for hCG-binding indicated that diluted (1:100, 1:200, 1:400) serum from naive queens had mean OD values (± SD) of 0.050 ± 0.021, 0.031 ± 0.011 and 0.020 ± 0.006, respectively. Individual serum samples were classified as positive (+) for hCG-binding if mean OD values exceeded naive sera means +3 SD (1:100, 0.113; 1:200, 0.064; 1:400, 0.038). Sera from male cats and all naive queens were negative for hCG-binding (Table 2) while five of eight queens stimulated three and four times with eCG/hCG

Table 2. ELISA binding activity for exogenous gonadotropins (eCG, hCG, pFSH) of sera from male cats, naive queens and queens previously receiving one (1x) treatment with eCG/hCG[a]

Trt group	Animal no.	eCG binding Serum dilution 1:100	1:200	1:400	hCG binding Serum dilution 1:100	1:200	1:400	pFSH binding Serum dilution 1:100	1:200	1:400
Male	17	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-
Naive	9	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-	-
1x	9	-	-	-	-	-	-	-	-	-
	10	-	-	-	+	+	+	-	-	-
	11	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	+	-	-
	14	-	-	-	-	-	-	-	-	-

[a] Optical density (OD) values of serum samples > and ≤ OD value (+3 SD) of naive sera were classified as positive (+) or negative (-), respectively.

Table 3. ELISA binding activity for exogenous gonadotropins (eCG, hCG, pFSH) of sera from queens previously receiving three (3x) or four (4x) treatments with eCG/hCG[a]

Trt group	Animal no.	eCG binding Serum dilution 1:100	1:200	1:400	hCG binding Serum dilution 1:100	1:200	1:400	pFSH binding Serum dilution 1:100	1:200	1:400
3x	1	+	+	+	-	-	-	+	+	+
	2	+	+	+	-	-	-	+	+	+
	3	+	-	-	+	+	+	-	-	-
	4	+	+	+	+	+	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	6	+	+	+	+	+	+	-	-	-
	7	+	+	+	+	+	+	-	-	-
	8	+	+	+	+	+	+	-	-	-
4x	1	+	+	+	-	-	-	+	+	+
	2	+	+	+	-	-	-	+	+	+
	3	-	-	-	+	+	+	-	-	-
	4	+	+	+	+	+	+	-	-	-
	5	-	-	-	-	-	-	-	-	-
	6	+	+	+	+	+	+	-	-	-
	7	+	+	+	+	+	+	-	-	-
	8	+	+	+	+	+	+	+	-	-

[a] Optical density (OD) values of serum samples > and ≤ OD value (+3 SD) of naive sera were classified as positive (+) or negative (-), respectively.

were positive (Table 3). Naive sera, diluted 1:100, 1:200 and 1:400, also was assessed for pFSH-binding activity, with mean OD values (± SD) of 0.329 ± 0.235, 0.216 ± 0.131 and 0.151 ± 0.069, respectively. Individual serum samples were considered positive (+) if the mean OD value was greater than the naive sera mean +3 SD (1:100, 1.034; 1:200, 0.609; 1:400, 0.358). While sera from male cats and all naive queens were negative for pFSH-binding (Table 2), sera from two of eight queens stimulated three times and three of eight queens stimulated four times with eCG/hCG were positive for pFSH-binding activity (Table 3).

Mouse Ovarian Stimulation Assay

In the pre-ovulatory mouse ovarian stimulation assay, mice injected with eCG/+sera had lower ($P<0.05$) ovary weights than mice injected with eCG/saline but similar ($P>0.05$) ovary weights to mice injected with saline alone (Figure 1). In the post-ovulatory mouse ovary stimulation assay, ovary weight only differed ($P<0.05$) among treatments for the saline control group (Figure 2a). Mice injected with eCG/+sera had fewer ($P<0.05$) ovulated oocytes than mice injected with eCG/-sera but this value did not differ ($P>0.05$) from mice injected with eCG/saline (Figure 2b). Within both assays, mean mouse weight did not differ ($P>0.05$) among treatment groups.

Discussion

While eCG/hCG combination regimens have proven invaluable for the application of assisted reproductive technology to endangered cat populations, the results of the present study indicate that the repeated administration of these gonadotropins may induce undesirable immunological consequences in cats. In this study, domestic cats repeatedly stimulated with eCG/hCG , at short intervals, demonstrated a significant decrease in both ovarian follicular development and the maturity of oocytes recovered from these follicles. Data from ELISA and mouse bioassays indicated that this quantitative and qualitative

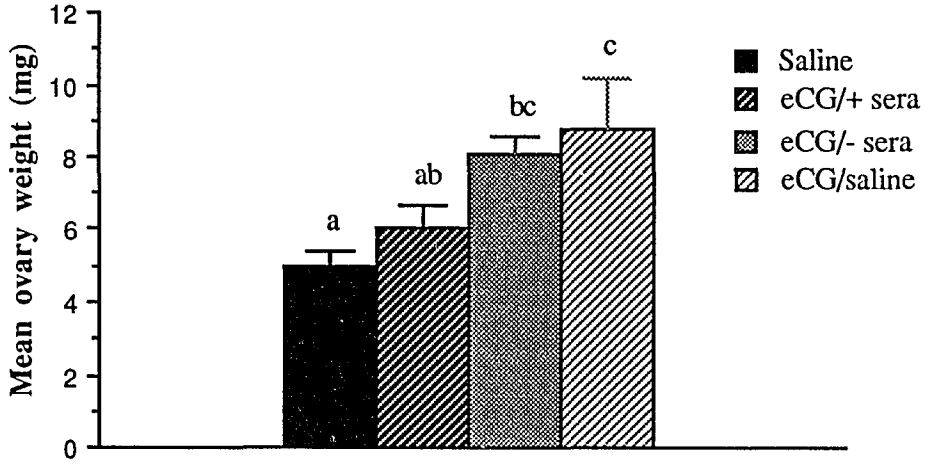

Figure 1. Pre-ovulatory mouse ovarian stimulation assay: Mean (± SEM) paired ovary weights of mice (n=10/trt) 48 hours following injection with either saline, eCG/+ sera, eCG/- sera or eCG/saline. Values with different superscripts are significantly different ($P<0.05$).

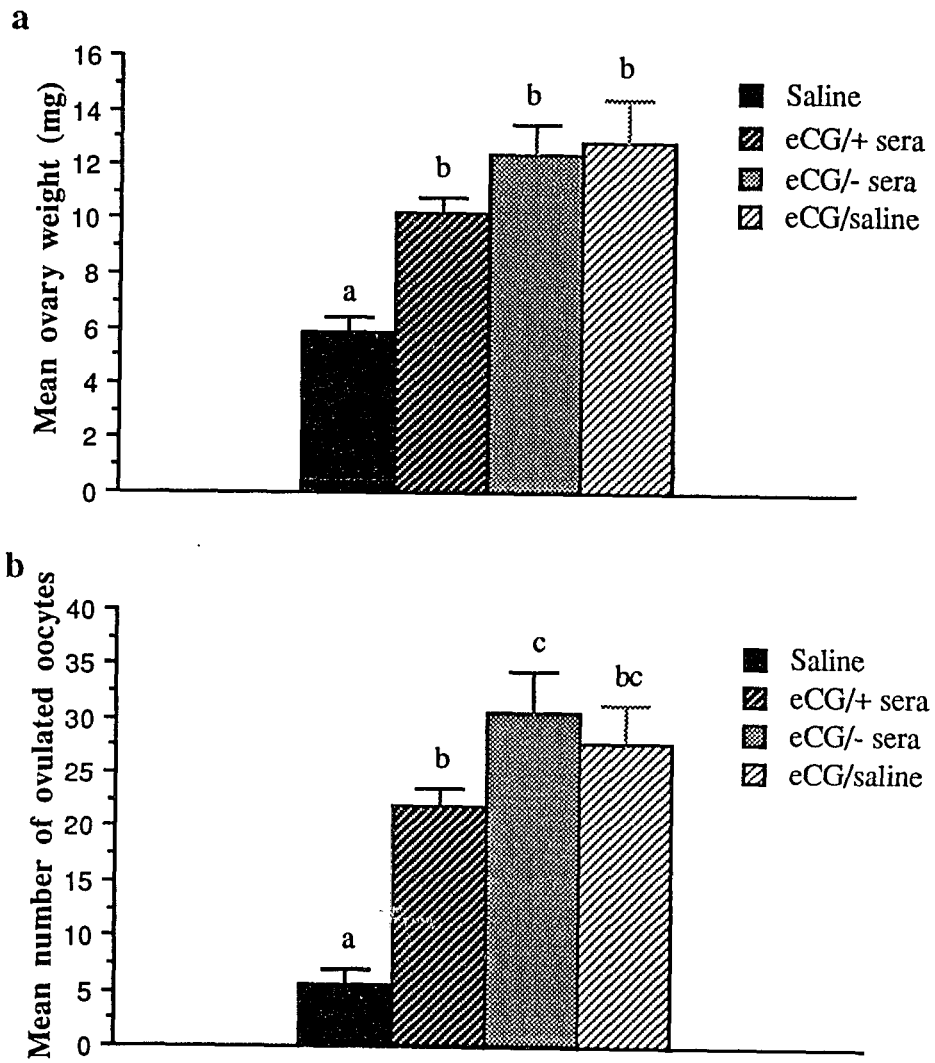

Figure 2. Post-ovulatory mouse ovarian stimulation assay: (a) Mean (± SEM) paired ovary weights of mice (n=12/trt) 20 hours following injection with hCG (5 IU). Mice were treated 47 hours prior to hCG with either saline, eCG/+ sera, eCG/- sera or eCG/saline. (b) Mean (± SEM) number of ovulated oocytes recovered from mice (n=12/trt) 20 hours following hCG injection. Values with different superscripts are significantly different ($P<0.05$).

deterioration in gonadotropin-induced folliculogenesis was a probable consequence of immunological interference with the bioactivity of eCG and/or hCG.

For this study, queens were initially stimulated with eCG/hCG at 44- to 50-day intervals to approximate the normal interestrous interval and to simulate a natural pattern of ovarian cyclicity. Gonadotropin-stimulated queens form functional corpora lutea (CL) following follicular aspiration (Goodrowe et al., 1988a; Donoghue et al., 1992b) and these CL have a comparable secretory lifespan to CL formed following natural mating with vasectomized males (Goodrowe et al., 1988a). Following sterile mating, queens typically exhibit a luteal phase (pseudopregnancy) duration of 36-38 days and an interestrual interval of 40-50 days (Paape et al., 1975; Wildt et al., 1981). Although the stimulation interval mimicked natural reproductive processes, queens exhibited a pronounced decrease in ovarian responsiveness following the third eCG/hCG treatment and this reduced responsiveness was not alleviated by lengthening the treatment interval. Given the physiochemical properties of eCG and hCG, an immunological basis was suspected as the cause of this ovarian refractoriness.

In cats, eCG and hCG are used in ovarian stimulation protocols for IVF and AI procedures because of their FSH-like and LH-like activities, respectively. Cats are induced ovulators (Gruelich, 1934) and, in the absence of natural mating, require an exogenous LH-like stimuli (such as hCG) to complete final ovarian follicular maturation (Goodrowe et al., 1988a; Donoghue et al., 1992b). In cats (especially easily stressed nondomestic species), eCG and hCG are preferred for ovarian stimulation protocols because these gonadotropins require only a single injection to exert their desired effects. The efficiency of a single injection is associated with both proteins having large carbohydrate moieties (Birken and Canfield, 1978; Pierce and Parsons, 1981) and, as a consequence, long elimination half-lifes and prolonged persistence in circulation after injection. The elimination half-life for eCG have been reported to range from 22 hours (sheep) to 51 hours (cattle) and, for hCG, from 48 minutes (rat) to 24 hours (primates) (McIntosh et al.,

1975; Menzer and Schams, 1979; Kaylan et al., 1982; Stouffer et al., 1986). In domestic cats, preliminary data (Swanson, unpublished) indicated an elimination half-life for eCG of approximately 24-48 hours following intramuscular injection. While the persistence of eCG and hCG in circulation is advantageous for ensuring their availability for FSH and LH receptor binding in the ovary, these long elimination half-lifes also prolong the exposure of antigenic sites on these foreign proteins to the immune system of the host.

Based on ELISA results, most queens treated multiple times with eCG/hCG generated both eCG and hCG -binding immunoglobulins. This finding is not surprising since both eCG and hCG are large (eCG, 45-65kD MW; hCG, ~37kD MW), immunologically complex proteins. Structurally, both glycoproteins are comprised of noncovalently linked alpha and beta subunits, with the major difference in bioactivity attributed to the nonhomologous portions (~30%) of the beta subunit (Birken and Canfield, 1978; Pierce and Parsons, 1981). While it appears that eCG was more consistently immunogenic after a few injections, given the homologous nature of these gonadotropins, some immunoglobulins may have expressed affinity for common epitopes on both eCG and hCG.

The cross-reactivity of specific sera for different gonadotropins also reflected individual variability in the immune responses of stimulated queens. Sera that were positive for eCG-binding were cross-reactive for hCG or pFSH binding but not for both gonadotropins. The selective cross-reactivity of immunoglobulins to shared epitopes between eCG and hCG (possibly) or eCG and pFSH demonstrates that the immune systems of individual queens may exhibit variable recognition of the multiple antigenic sites found on exogenous gonadotropins. This variability may be one factor in developing approaches to mitigate the development of ovarian refractoriness in individual queens.

The capacity of these gonadotropin-binding immunoglobulins to affect exogenous gonadotropin activity *in vivo* was evaluated in pre- and post-ovulatory mouse ovarian stimulation assays. Collectively, results of these assays indicated an overall decreased

responsiveness of mice when treated with eCG mixed with eCG/hCG antisera. This observation is important since exogenous gonadotropins may have separate biological and immunological active sites (Christakos and Bahl, 1979) and mere binding of immunoglobulins does not always neutralize biological activity (Louvet et al., 1974). In retrospect, a more pronounced difference between treatment groups may have resulted if the volume of positive sera relative to the amount of injected eCG (10 µl sera/IU eCG) was increased to more closely approximate physiological levels seen in immunorefractory domestic cats (~1000 µl sera/IU of injected eCG; based on estimated blood volume in cats and the standard eCG dosage).

Of utmost importance, treatment of eCG/hCG refractory queens with an alternative ovarian stimulation protocol, using pFSH, demonstrated that the ovaries of these queens are still responsive to exogenous folliculogenic stimuli. None of these queens had sera with high levels of anti-pFSH immunoglobulins prior to pFSH treatment and, consequently, they developed significantly more follicles than during the three previous eCG/hCG stimulation procedures. Maturity of recovered oocytes was still decreased, possibly due to impairment in hCG bioactivity, since five of these six queens had positive sera for hCG-binding immunoglobulins. Because control queens (i.e., non-refractory) were not stimulated simultaneously, definite conclusions about oocyte maturity are not possible. However, a similar pFSH/hCG regimen (Pope et al., 1993a) has been assessed in an IVF protocol for domestic cats and, in that study, 97% of recovered oocytes were classified as mature. The responsiveness of refractory queens to pFSH supports the assertion that immunological interference is inhibiting ovarian responsiveness to eCG/hCG but also indicates that alternative gonadotropin stimulation protocols might be used in refractory queens, provided immunological cross-reactivity is minimal. This finding is critically important for nondomestic felids, since individual cats of a species may be irreplaceable from a genetic perspective (unrepresented founders, for example) (Foose,

1983) but, due to physiological or logistical constraints, may require intensive management for assisted reproduction purposes.

Also of major importance, natural cyclicity did not appear to be inhibited by the presence of eCG/hCG neutralizing immunoglobulins. Starting 1 to 2 months following the fourth stimulation attempt, signs of behavioral estrus were detected in seven of eight immunorefractory queens and laparoscopy of four of these estrual queens revealed the presence of multiple developing follicles on their ovaries (data not shown). The occurrence of natural cyclicity, despite the presence of gonadotropin-neutralizing gonadotropins, is consistent with findings in other species (Lin and Bailey, 1965; Jainudeen et al., 1966; Land and McLaren, 1967; Greenwald, 1970; Reel et al., 1976; Bavister et al., 1986). Immunoglobulins directed against exogenous gonadotropins frequently are specific for epitopes unique to those proteins, with minimal cross-reactivity for endogenous gonadotropins (FSH, LH). In the present study, endogenous FSH bioactivity appeared unaffected but the impact of circulating immunoglobulins on endogenous LH function and ovulation induction following natural mating was not assessed. Especially for endangered nondomestic species, it is essential that assisted reproductive techniques do not compromise the future potential of animals to bear offspring naturally.

While studies in other species also have reported ovarian refractoriness to repeated exogenous gonadotropin administration, few of these studies have attempted to systematically investigate the nature of the immunological response and examine its specific impact on gonadotropin-induced reproductive function. Furthermore, these immunological consequences have never been investigated in cats, despite the increasing use of eCG/hCG combination regimens in domestic and endangered nondomestic cats and the potential implications for these species. The advantages of these exogenous gonadotropins, in inducing desirable physiological responses (after a single injection), make their continued use imperative for the future reproductive management of genetically-valuable or endangered cat populations. However, immunological realities dictate that these

gonadotropin combinations should be used judiciously in cats and that the suitability of alternative ovarian stimulation protocols should receive additional investigation.

CHAPTER III

KINETICS OF THE HUMORAL IMMUNE RESPONSE FOLLOWING MULTIPLE TREATMENTS WITH EXOGENOUS GONADOTROPINS AND RELATIONSHIP TO OVARIAN RESPONSIVENESS IN THE DOMESTIC CAT

Introduction

Assisted reproductive technology, such as *in vitro* fertilization (IVF) and artificial insemination (AI), has tremendous potential for the captive management and conservation of genetically valuable and/or biologically endangered cat populations (Wildt, 1990; Wildt et al., 1992b). As one component of these assisted reproductive techniques, combination regimens of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) have proven highly effective for stimulating ovarian follicular development and inducing follicular maturation or ovulation, respectively. Using these combination regimens, the application of AI and IVF (with embryo transfer) procedures has allowed the production of viable offspring in domestic cats (Goodrowe et al., 1988a; Howard et al., 1992a; Howard et al., 1993b; Swanson and Godke, 1994a) and in a number of nondomestic cat species (Donoghue et al., 1990; Howard et al., 1992b; Donoghue et al., 1993a; Barone et al., 1994).

One potential complication, however, is that the repeated use of combination regimens has been associated with the development of gonadotropin-neutralizing immunoglobulins and ovarian refractoriness to subsequent gonadotropin-induced stimulation in domestic cats and other species (Lin and Bailey, 1965; Maurer et al., 1968; Swanson et al., 1994b). In a previous study in cats (Swanson et al., 1994b), queens treated twice with a standard eCG/hCG regimen (Johnston et al., 1991a,b; Donoghue et al., 1992b) at a short interval (44-50 days) became resistant to further gonadotropin-induced folliculogenesis on the third stimulation attempt. While sera collected from gonadotropin-refractory queens were shown to contain eCG/hCG-neutralizing immunoglobulins, the kinetics of immunoglobulin production following the initial and

subsequent eCG/hCG injections and the relationship of immunokinetics to ovarian response was not investigated.

Both eCG and hCG are large (30-70 kD MW) immunologically-complex glycoproteins (Birken and Canfield, 1978; Pierce and Parsons, 1981) so the native proteins might be expected to induce typical primary and secondary immunoglobulin responses with short injection intervals in cats. By prolonging the interval between successive treatments, however, a pronounced anamnestic immunological response might be avoided and queens possibly would remain responsive to subsequent eCG/hCG treatments. Because these eCG/hCG combination regimens are extremely valuable in the assisted reproduction of felid species, additional studies are needed to further clarify immunological responses and to develop approaches that may mitigate potential immunological complications. Accordingly, the objectives of the present study were to compare ovarian responses (follicle development, oocyte maturity) of queens repeatedly stimulated at short versus long eCG/hCG stimulation intervals and to investigate the kinetics of eCG-binding immunoglobulin production in relation to the observed ovarian responses.

Materials and Methods

Experimental Animals

Random-source, adult female domestic cats (n=11) were conditioned as previously described (Swanson et al., 1994b) and housed singly or in pairs in stainless steel cages. Queens were maintained under a controlled (12 hours light:12 hours dark) artificial illumination environment and were provided a commercial cat food diet (Science Diet, Hills Pet Products, Topeka, KS) and water *ad libitum*.

Gonadotropin Administration and Laparoscopy

Gonadotropin-induced ovarian stimulation and laparoscopy were as previously reported (Swanson et al., 1994b), based on earlier studies (Wildt et al., 1977; Goodrowe et al., 1988a; Johnston et al., 1991a,b). Briefly, anestrual queens were injected with 150 IU

eCG (Sigma Chemical Company, St. Louis, MO; i.m.) followed 84 hours later with an injection of 100 IU hCG (Sigma; i.m.). Queens were anesthetized at 24-27 hours post-hCG and their ovaries were examined at laparoscopy for the presence of mature vesicular follicles (≥2 mm in diameter). Mature follicles were transabdominally aspirated and the recovered oocytes were assessed for maturational status. Mature oocytes possessed distinct corona radiata and expanded cumulus cell masses and immature oocytes had tightly compacted cumulus cell investments.

Naive queens (n=11) were randomly assigned to two treatment groups: 1) short interval (49-57 days between eCG/hCG treatments) and 2) long interval (130-135 days between eCG/hCG trreatments). Queens in both groups were administered exogenous gonadotropins and subjected to laparoscopy three consecutive times at the interval appropriate for each group. In addition, queens in the long interval group were treated a fourth time 66-67 days following the third stimulation. Blood was collected from all naive queens via jugular venipuncture 1-4 weeks prior to the first eCG/hCG treatment. Blood also was collected from queens (n=6) in the short interval group at the time of each laparoscopy and every 6-8 days during each treatment interval until 6 weeks following the third stimulation. Blood was obtained from queens (n=5) in the long interval group at the time of each laparoscopy, one week prior to the second eCG/hCG treatment and then every 6-8 days during each subsequent treatment interval until 4 weeks after the fourth stimulation. Because anamnestic immune responses were of primary interest (and to lessen stress on study animals), blood samples were not collected weekly from queens in the long interval group during the first treatment interval. All sera was stored frozen at -20°C until analyzed by ELISA.

Solid-phase ELISA

ELISA procedures were similar to those previously described (Swanson et al., 1994b) with only slight modification. Briefly, eCG (Sigma) was diluted in 60 mM carbonate-bicarbonate buffer (pH 9.6) and aliquots (25 µl) were pipetted into wells (500 ng

protein/well) of microtiter plates (Immulon I, Dynatech Laboratories, Alexandria, VA). Plates were incubated for 3 hours at 37°C, washed five times with 0.01M phosphate-buffered saline- 0.1% Tween 20 (PBS-Tw) and blotted dry. Thawed serum samples were serially diluted (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200) with PBS-Tw, aliquots (100 µl) were added to wells and plates were incubated for 1 hour at room temperature (22°C).

Following plate washing, horseradish peroxidase (HRP)-conjugated, affinity purified rabbit anti-cat IgG (Zymed Laboratories, South San Francisco, CA) was added (100 µl ; 1:1000 dilution in PBS-Tw) to appropriate wells and plates were incubated for 30 minutes at 37°C. After a final plate washing, 100 µl of *o*-phenylenediamine solution (Sigma; 1 mg/ml 0.05 M citrate buffer, pH 4.5) with 0.05% H_2O_2 was added to wells and plates were incubated in the dark for 40 minutes at room temperature. The color reaction in each well was stopped with 50 µl of 2.5 M H_2SO_4 and the optical density (OD) was measured at 492 nm using an automated microplate reader (MR5000 model, Dynatech).

Different commercial lots of eCG were used for ELISA and for treatment of queens. Serum samples from each interval group were equally distributed among microtiter plates and each plate included a negative serum reference (1:100 dilution), comprised of pooled serum from naive queens, in addition to substrate, serum and enzyme conjugate controls. Mean OD values (± SD) were determined for the triplicate wells of the negative serum reference. Within each plate, OD values of serum samples above the mean value for the negative serum reference +3 SD (for all negative reference wells) were considered positive for the presence of eCG-binding immunoglobulins. The end-point titer was expressed as the inverse (x 10^{-2}) of the highest dilution that was positive and serum samples that were negative at every dilution were assigned a titer of 0 (i.e., <1:100).

Statistical Analysis

The mean number (± SEM) of follicles (≥ 2 mm in diameter) and the proportion of recovered oocytes classified as mature were calculated for queens in short and long interval groups. Follicle numbers within groups were evaluated using analysis of variance and

differences between means determined with a least significance difference (LSD) test (SAS, 1984). The proportion of mature oocytes for each treatment period was compared using Chi square analysis (Steel and Torrie, 1960). The influence of specific serum titers on number of follicles and proportion of queens with >10 follicles/treatment was evaluated with a Students *t*-test and a Fisher exact test (Steel and Torrie, 1960), respectively.

Results

Ovarian Responses for Short and Long Stimulation Intervals

Queens repeatedly treated with exogenous gonadotropins at short intervals (Table 4) tended to exhibit fewer follicles with each successive eCG/hCG treatment. Following the third treatment, the number of follicles observed were less ($P<0.05$) than for the first treatment and the proportion of recovered oocytes that were mature was less ($P<0.05$) than for the second treatment. In contrast, queens treated at long intervals (Table 5) exhibited no change ($P>0.05$) in follicle number or proportion of mature oocytes produced in response to the first three stimulation procedures. Furthermore, a fourth eCG/hCG treatment at a 66- to 67-day interval resulted in a similar ($P>0.05$) mean number of follicles and proportion of mature oocytes compared to the first three treatments.

Serial Serum Titers of eCG-Binding Immunoglobulins

Results of the ELISA indicated that negative serum reference wells had a overall mean OD value ($\pm$ SD) of 0.284 ± 0.077 with an intra-assay and inter-assay coefficient of variation (c.v.) of 12% and 25%, respectively. Sera from 10 of 11 naive queens were negative (0 titer) for eCG-binding activity, based on having OD values (at all dilutions) less than the OD value for each plate-specific negative serum reference +3 SD. Serum from one naive queen (Cat No. 36) was marginally positive (at a titer of 1) on two separate repetitions of the assay.

Queens in the short interval group (Figure 3) showed variable primary and secondary immune responses following eCG/hCG treatment. Four of six queens (Cat No.

Table 4. Effect of repeated eCG/hCG administration at short intervals (49-57 days) on number of ovarian follicles and production of mature oocytes in the domestic cat[a]

Animal number	Number of follicles observed with sequential eCG/hCG treatments		
	1st treatment	2nd treatment	3rd treatment
43	29	22	2
44	17	0	0
45	22	13	3
46	20	18	14
47	11	16	12
48	16	4	2
Mean number (± SEM) of follicles/queen	18.0 ± 1.7[b]	13.3 ± 4.2[bc]	5.5 ± 2.4[c]
Proportion (%) of oocytes classified as mature	79/85 (92.9%)[bc]	59/61 (96.7%)[b]	27/32 (84.3%)[c]

[a] Queens were treated with an eCG/hCG regimen at 49- to 57-day intervals for three consecutive treatments. With each treatment, laparoscopy was conducted 24-27 hours post-hCG and ovarian follicles were aspirated to recover oocytes.

[b-c] Values within rows with different superscripts are significantly different ($P<0.05$).

Table 5. Effect of repeated eCG/hCG administration at long intervals (130-135 days) on number of ovarian follicles and production of mature oocytes in the domestic cat[a]

Animal number	Number of follicles observed with sequential eCG/hCG treatments			
	1st treatment	2nd treatment	3rd treatment	4th treatment
34	15	16	6	4
35	9	2	10	13
36	25	27	54	39
37	7	7	16	15
38	10	18	11	24
Mean number (± SEM) of follicles/queen	13.2 ± 5.2	14.0 ± 3.8	19.4 ± 8.8	19.0 ± 5.9
Proportion (%) of oocytes classified as mature	53/58 (91.4%)[bc]	48/54 (88.9%)[b]	83/85 (97.6%)[c]	79/84 (94.0%)[bc]

[a] Queens were treated with an eCG/hCG regimen at 130- to 135-day intervals for the first three treatments and at a 66- to 67-day interval for the fourth treatment. With each treatment, laparoscopy was conducted 24-27 hours post-hCG and ovarian follicles were aspirated to recover oocytes.

[b-c] Values within rows with different superscripts are significantly different ($P<0.05$).

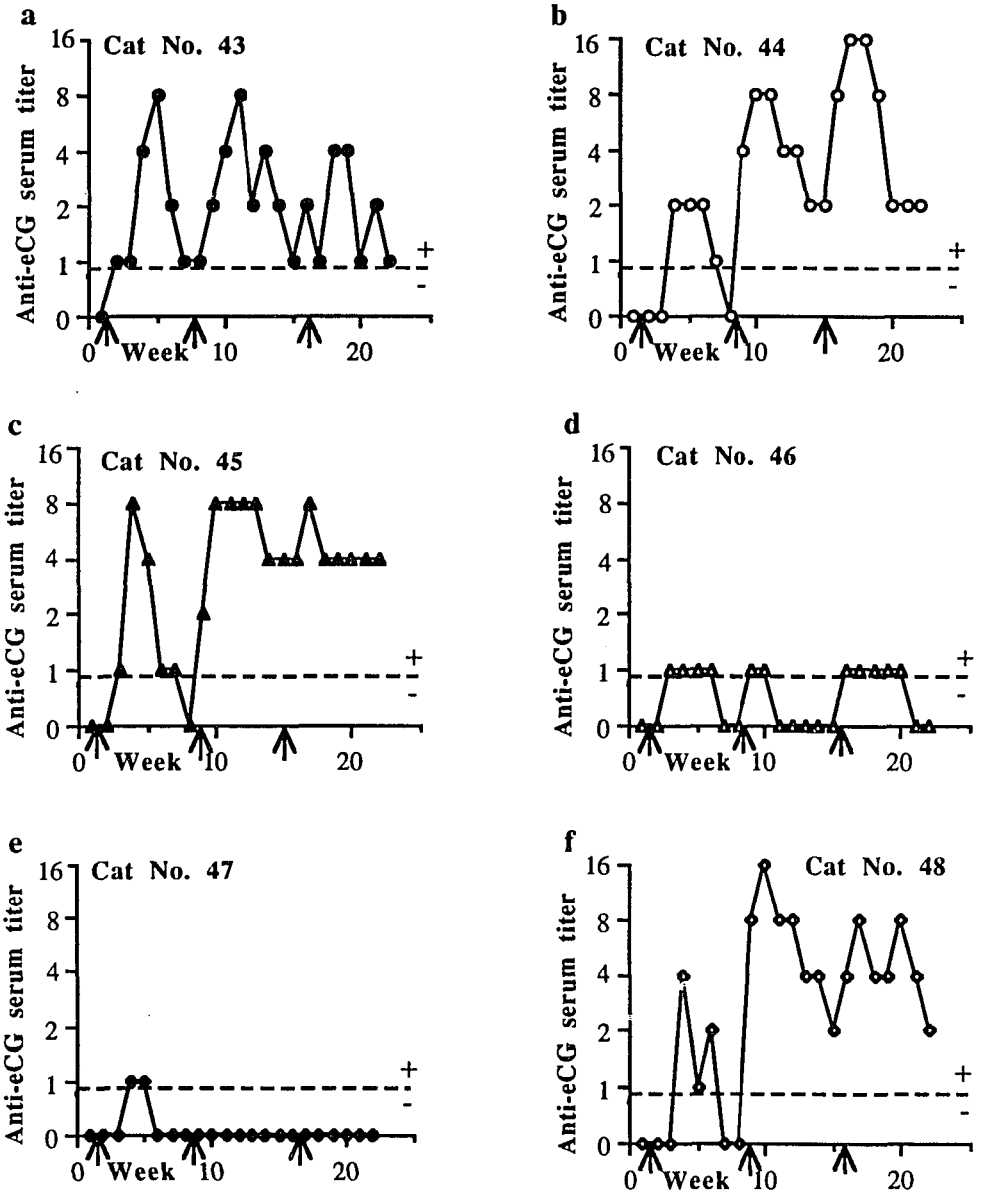

Figure 3. Serial eCG-binding immunoglobulin titers (x 10^{-2}) for queens treated three times with exogenous gonadotropins (eCG/hCG) at short intervals (49-57 days): a) Cat No. 43; b) Cat No. 44; c) Cat No. 45; d) Cat No. 46; e) Cat No. 47; and f) Cat No. 48. Arrows indicate the time of eCG injection with serum titers at laparoscopy represented by the next data point after each arrow. The dashed horizontal line indicates the cut-off point between positive and negative sera.

43, No. 44, No. 45 and No. 48; Figure 3a, 3b, 3c and 3f, respectively) exhibited two- to four-dilution increases in serum titers within 2-4 weeks of eCG/hCG injection and titers remained elevated above baseline levels (0 titer) for at least 2-6 weeks. After the second eCG/hCG treatment, these four queens had three- to four-dilution elevations in serum titers with a shorter latent period (≤1 week) and titers were still elevated at the time of the third eCG/hCG stimulation. Following the third injection, serum titers increased moderately over the next 1-2 weeks. In contrast, two queens (Cat No. 46 and No. 47; Figure 3d and 3e, respectively) had minor one-dilution elevations of serum titers 2-3 weeks following the initial eCG/hCG injection and either no or minor (one-dilution) increases in titers after the second and third eCG/hCG treatment.

Queens in the long interval group (Figure 4) displayed immune responses with a similar variability but generally of a decreased magnitude compared to queens in the short interval group. The titer of one queen (Cat No. 38; Figure 4e) rapidly increased two dilutions between initial eCG/hCG treatment and laparoscopy, but the titers of the other four queens did not change during this short time period. At the time of the second eCG/hCG injection, four queens had basal titers and one queen had a low positive titer. Following the second eCG/hCG treatment, three queens (Cat No. 34, No. 36 and No. 37; Figure 4a, 4c and 4d, respectively) had two- to three-dilution elevations in titers within 2 weeks but peak serum titers were lower than that observed in strongly-responsive queens in the short interval group. After the third eCG/hCG injection, one of these queens (Cat No. 34, Figure 4a) had a rapid (<2 weeks) three-dilution increase in serum titer but the other two cats exhibited only minor titer elevations. The other two queens (Cat No. 35 and No. 38; Figure 4b and 4e, respectively) showed no or only minor changes in titers following the second and third eCG/hCG injections. Following the fourth eCG/hCG treatment (at a shorter interval of 66-67 days), no queen exhibited more than a one-dilution increase in serum titer.

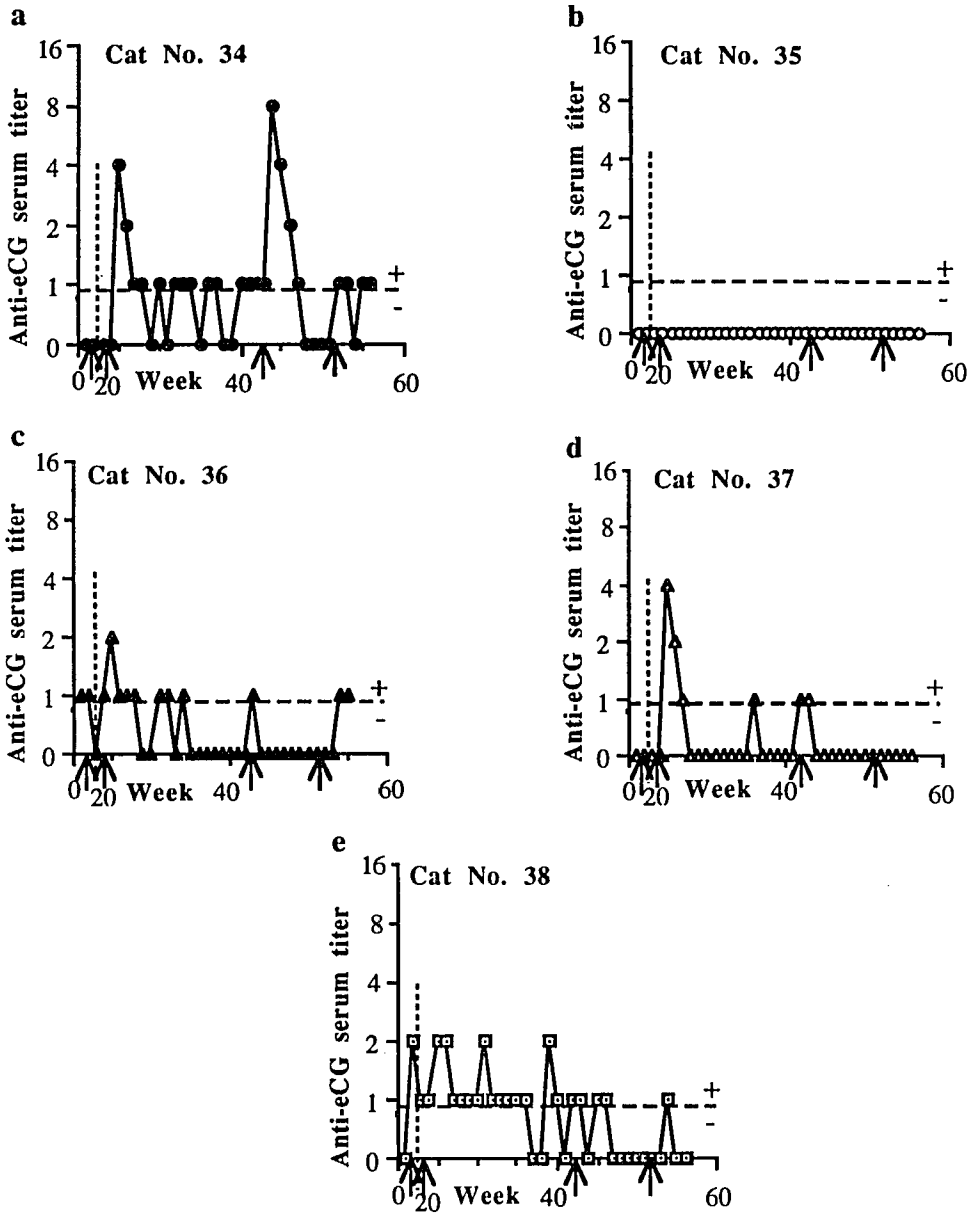

Figure 4. Serial eCG-binding immunoglobulin titers (x 10^{-2}) for queens treated three times with exogenous gonadotropins (eCG/hCG) at long intervals (130-135 days): a) Cat No. 34; b) Cat No. 35; c) Cat No. 36; d) Cat No. 37; and e) Cat No. 38. Arrows indicate the time of eCG injection with serum titers at laparoscopy represented by the next data point after each arrow. The dashed vertical line represents the first stimulation interval (in which no blood samples were collected) and the horizontal line indicates the cut-off point between positive and negative sera. The third stimulation interval was shorter (66-67 days) than the previous two intervals.

Comparative analysis of ovarian responses and serum titers at the time of eCG injection and laparoscopy indicated that serum titers ≤1 had a positive influence on the number of follicles and the proportion of queens with >10 follicles. Queens with a serum titer of ≤1 at both eCG injection and laparoscopy produced an average of 16.8 ± 1.9 follicles and 75.9% (22/29) of these stimulation attempts resulted in >10 ovarian follicles. Queens with a serum titer ≥ 2 at eCG injection or at laparoscopy produced fewer ($P<0.05$) follicles (7.0 ± 3.1) and a smaller ($P<0.05$) percentage (22.2%; 2/9) of these stimulation attempts produced >10 ovarian follicles.

Discussion

The results of the present study support earlier findings (Swanson et al., 1994b) of ovarian and humoral immunological responses in queens treated repeatedly, at short intervals, with an eCG/hCG combination regimen. In both studies, the number of ovarian follicles and the maturity of oocytes recovered from these follicles decreased with the third successive gonadotropin treatment and sera from refractory queens typically contained elevated levels of eCG-binding immunoglobulins. These immunoglobulins were shown to exhibit variable cross-reactivity with hCG and porcine follicle stimulating hormone (pFSH) and to neutralize or attenuate eCG/hCG bioactivity in the mouse (Swanson et al., 1994b). In the present study, the kinetics of anti-eCG immunoglobulin generation and the relationship of individual immunological responses to ovarian refractoriness were characterized.

In the short interval group, all queens responded to the first eCG/hCG treatment by producing multiple ovarian follicles with a high proportion of recovered oocytes classified as mature. Four of these queens developed peak eCG-binding immunoglobulin titers 2-3 weeks following injection in a relatively long latent period typical of primary immunoglobulin responses. Because the enzyme conjugate used in the ELISA was affinity-purified to the heavy and light chains of IgG, the IgM component of the primary

response was probably minimally cross-reactive in the ELISA (Mitani et al., 1988). Presumably, the detected elevation in anti-eCG immunoglobulins was primarily IgG so that the true magnitude of the primary immune response probably was understated. The secondary responses of these four queens were fairly typical, with shorter latent periods, the same or higher peak titers and a longer steady state. In two of these queens (Cat No. 44 and No. 48), serum titers increased rapidly from basal levels between the time of eCG injection and laparoscopy. Interestingly, these queens produced only 0 and 4 follicles on their ovaries, respectively, suggesting that the anamnestic responses may have been strong enough to interfere with eCG/hCG activity during the second ovarian stimulation attempt. Subsequently, at the third eCG injection, these queens still showed elevated immunoglobulin levels and, at laparoscopy, only 0 and 2 ovarian follicles were observed, respectively.

The other two immunoresponsive queens in the short interval group (Cat No. 43 and No. 45) had rapid but less pronounced anamnestic responses following the second gonadotropin treatment and developed 22 and 16 ovarian follicles, respectively. However, serum titers were still elevated when the third eCG injection was administered and these two queens subsequently developed just 2 and 3 follicles, respectively. In contrast, the final two queens in the short interval group (Cat No. 46 and No. 47) had minimal primary and secondary responses to eCG/hCG injection, never developed high serum titers (>1) and, following each eCG/hCG treatment, consistently developed >10 ovarian follicles and a high proportion of mature oocytes. These results agree with the previous study (Swanson et al., 1994b) indicating that most cats apparently become refractory to eCG/hCG-induced ovarian stimulation following one or two gonadotropin treatments. Similarly, rabbits developed immunological refractoriness after one or two eCG/hCG treatments when administered at short intervals (8 weeks) (Maurer et al., 1968) but mice required four or more treatments before ovarian responses declined (Lin and Bailey, 1965). These results also suggest an inverse relationship between the rapidity and amplitude of the

humoral immune response and the capacity of queens to produce multiple ovarian follicles following eCG/hCG treatment. Results from queens in the long interval group support this conclusion.

Queens in the long interval group did not demonstrate reduced follicular production or oocyte maturity with repeated gonadotropin treatments. Immunologically, three of five queens (Cat No. 34, No. 36 and No. 37) showed evidence of secondary responses with rapid elevations in serum titers but not to levels observed for the refractory queens in the short interval groups. Titers usually decreased quickly and these cats remained responsive to gonadotropin treatment, even when a fourth, shorter treatment interval was used. The two remaining queens (Cat No. 35 and No. 38) had minimal immunological responses to eCG/hCG injection during the entire study period and also remained consistently sensitive to eCG/hCG-induced ovarian stimulation. These results suggest that long intervals (130-135 days) between exogenous gonadotropin administration are effective in preventing the development of ovarian refractoriness in the cat. In contrast to cats, rhesus monkeys are unresponsive to a second eCG treatment when administered alone for ovarian stimulation at 5 or 6 month intervals (Bavister et al., 1986). This variability in immune responses between species and between individual animals within a species obviously should be an important consideration in the design and application of ovarian stimulation protocols.

Genetic variation has been reported in the development of ovarian refractoriness in rabbits to hCG (Greenwald, 1970) and in two strains of mice to eCG/hCG (Lin and Bailey, 1965), presumably due to variable individual or strain-specific sensitivity of immune responses. In some queens, shorter stimulation intervals may not be contraindicated if their immune systems consistently fail to recognize eCG and hCG as foreign proteins but, in general, longer intervals between gonadotropin stimulations are probably preferable for outbred heterozygous cat populations. In some nondomestic felid species, however, population bottlenecks and/or inbreeding have greatly reduced genetic heterozygosity and possibly decreased the potential variability of immunological responses. For example, the

world's population of cheetahs is essentially genetically monomorphic and exhibits a similar exaggerated susceptibility to certain infectious agents (O'Brien et al., 1983; O'Brien et al., 1985). This genetic and immunological uniformity could affect the general suitability and acceptable frequency of exogenous gonadotropin treatment for assisted reproduction in some cat species. Preliminary data (Swanson et al., unpublished) in the cheetah, however, indicates that this species is not immunologically hypersensitive to eCG/hCG administration and will remain responsive to the ovarian stimulatory effects of exogenous gonadotropins following multiple treatments at 6-month intervals. However, the impact of shorter intervals between gonadotropin treatments on ovarian and immunological responses have not been investigated in cheetahs or any other cat species.

Of more practical application, queens in genetically-valuable domestic cat populations might be screened for titer levels of eCG-binding immunoglobulins and, based on the predicted ovarian responses associated with certain serum titers, queens with titers of 2 (i.e., positive at 1:200 dilution) or higher would not be stimulated with eCG/hCG. For nondomestic cat species, a similar screening procedure could be particularly useful. Although domestic cats are valuable research models for nondomestic species (Wildt et al., 1986), species differences in ovarian responsiveness to exogenous gonadotropins requires that ovarian stimulation protocols still must be refined for each separate cat species (Howard et al., 1993a). Individual and species variability in immune responses and differences between species in required gonadotropin dosages likely would affect the specific kinetics of anti-gonadotropin immunoglobulin production. Because of these potential differences, a conservative approach to screening of nondomestic species would be to exclude any cat with even a minimal anti-gonadotropin titer.

Sera from refractory cats also could be assayed for cross-reactivity with other gonadotropins (hCG, pFSH) to aid in the selection of potential alternative strategies for ovarian stimulation. High eCG-binding immunobulin titers are usually coupled with elevations in either anti-hCG or anti-pFSH titers but not both (Swanson et al., 1994b).

The nature of the antisera cross-reactivity would dictate whether regimens employing alternative FSH- or LH-like signals would be required. However, more problematic in this approach to pre-stimulation screening are cats with basal anti-eCG titers that rapidly increase within a few days post-injection to inhibit gonadotropin activity. Based on pre-stimulation titers, these females might be expected to respond satisfactorily to the standard eCG/hCG regimen.

In conclusion, ovarian stimulation protocols, using exogenous gonadotropins such as eCG/hCG, are critically important in the assisted propagation of domestic and non-domestic felids (Wildt, 1990; Wildt et al., 1992b). The repeated treatment of domestic cats with these foreign proteins elicits variable humoral immune responses in individual cats but frequently induces the development of immunologically-mediated ovarian refractoriness. These immunological complications may be largely avoided in the domestic cat by lengthening the interval between successive gonadotropin injections and this information deserves serious consideration when developing assisted reproductive strategies for endangered felids. Still, the potential immunological consequences of unlimited repetitions of eCG/hCG treatment, even at longer treatment intervals, have not been determined.

CHAPTER IV

ANCILLARY FOLLICLE AND SECONDARY CORPORA LUTEA FORMATION FOLLOWING EXOGENOUS GONADOTROPIN TREATMENT IN THE DOMESTIC CAT AND EFFECT OF PASSIVE TRANSFER OF GONADOTROPIN-NEUTRALIZING ANTISERA

Introduction

In domestic cats, combination ovarian stimulation regimens of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) have permitted the generation of viable offspring when used in conjunction with *in vitro* fertilization (IVF) and embryo transfer (ET) (Goodrowe et al., 1988a; Swanson and Godke, 1994a) or artificial insemination (AI) procedures (Howard et al., 1992a). With these IVF/ET and AI techniques, however, embryo survival and/or pregnancy rates are generally low in exogenous gonadotropin-stimulated queens. This diminished fertility may be the result of intrinsic gamete or embryo abnormalities or may be a consequence of an inappropriate maternal environment. Following eCG/hCG treatment and ovarian follicular aspiration or AI, domestic cats have been shown to develop multiple ancillary ovarian follicles (Donoghue et al., 1992b) and accessory CL (Goodrowe et al., 1988a; Howard et al., 1992a) when evaluated 6 to 7 days post-aspiration (or AI). In addition, one nondomestic cat species, the tiger, has been reported to exhibit the formation of ancillary follicles following similar eCG/hCG stimulation and follicular aspiration procedures (Donoghue et al., 1990). Alterations in the endocrine mileau (i.e., progesterone and estradiol-17ß), possibly associated with these secondary ovarian structures, may affect hormonally-driven changes in the oviductal and uterine environment (Gidley-Baird et al., 1986) and, consequently, impact on embryo survival in these cats.

In cattle, superovulation protocols using eCG also have been associated with the development of additional ovarian follicles post-ovulation (Booth et al., 1975; Saumande, 1980) and these ancillary follicles have been implicated as one factor in the recovery of poor quality embryos from superovulated cows (Booth et al., 1975). Because the formation of ancillary follicles may be caused by the prolonged persistence of eCG

post-injection (Menzer and Schams, 1979), corrective strategies have involved injections of anti-eCG immunoglobulins to neutralize remaining eCG in circulation (Dieleman et al., 1993; for review). With proper timing of anti-eCG treatment, improvements have been reported in ovulation rate (Dieleman and Bevers, 1987), number of secondary follicles (Dieleman et al., 1989) and number of transferable quality embryos (Dieleman et al., 1989) in eCG-treated cows.

A similar approach to eCG-neutralization in domestic cats might be useful for decreasing the formation of secondary follicles and CL and potentially improving the maternal environment. An added benefit of passive immunoglobulin transfer and early eCG-neutralization might be attenuation of the humoral immune responses frequently observed in queens treated with eCG (Swanson et al., 1994a,b). Accordingly, the objectives of the present study were to: 1) obtain an estimation of the rate of elimination and persistence of eCG following intramuscular injection in domestic cats, 2) further characterize the development of secondary ovarian follicles and CL in eCG-stimulated queens following follicular aspiration and 3) evaluate the effect of exogenous gonadotropin-neutralizing antisera on secondary follicle and CL formation, CL function and humoral immune responses.

Materials and Methods

Experimental Animals

Random-source, adult female domestic cats (n=19) were conditioned and maintained as previously described (Swanson et al., 1994b). Cats were housed under a controlled artificial illumination cycle and were provided a nutritionally-balanced, commercial cat diet (Science Diet, Hill's Pet Products, Topeka, KS) and water *ad libitum*.

Exogenous Gonadotropin Stimulation and Laparoscopy

Gonadotropin-induced ovarian stimulation and laparoscopy followed previously described procedures (Swanson et al., 1994a,b), based on earlier studies (Wildt et al.,

1977; Goodrowe et al., 1988a; Johnston et al., 1991a,b). Briefly, anestrual queens were injected (i.m.) with 150 IU eCG (Sigma Chemical Company, St. Louis, MO) followed 84 hours later with an injection of 100 IU hCG (Sigma). At 24-27 hours post-hCG, anesthetized queens were examined by laparoscopy to determine the number of mature vesicular follicles (≥2 mm in diameter) and corpora lutea (CL) present on the ovaries. Mature follicles were transabdominally aspirated and recovered oocytes were assessed for maturational status, based on morphological characteristics (Swanson et al., 1994b). For a given queen, if the number of recovered oocytes exceeded the number of counted follicles, the follicle number was adjusted accordingly. Five and 15 days following follicular aspiration, queens were re-evaluated by laparoscopy to determine the number of ovarian structures (follicles, CL). Subsequently, queens were stimulated a second time with eCG/hCG (at a 44- to 45-day-interval between regimens) and again subjected to laparoscopy and follicular aspiration.

eCG Radioimmunoassay and Estimation of eCG Elimination

Blood samples were collected via jugular venipuncture from naive queens (n=3; body weight, 3.8-4.4 kg) just prior to intramuscular injection of eCG (150 IU) and then at 12, 36, 60 and 84 hours post-injection. Queens were administered hCG (100 IU, i.m.) and evaluated by laparoscopy 24-27 hours later. Blood samples also were collected at laparoscopy and 2, 4 and 6 days post-laparoscopy and sera was stored frozen at -20°C until analyzed. Radioimmunoassay (RIA) for eCG followed previously validated procedures (Thompson et al., 1982a,b). Serum samples (100 µl) were assayed in duplicate using rabbit anti-eCG antisera with radioiodinated ovine LH as the radiolabeled ligand and bound and unbound eCG were separated by precipitation with sheep anti-rabbit gamma globulin. Assay sensitivity was approximately 0.2 mIU of eCG (or 2 mIU/ml for a 100 µl sample) and intra-assay and inter-assay coefficients of variation were 8% and 12%, respectively. For this assay, cross-reactivity of rabbit anti-eCG antisera with hCG was low and nonparallel (Thompson, unpublished data); however, the elimination half-life of eCG was

estimated based only on changes in measured serum concentrations between 12 and 84 hours post-injection (i.e., prior to hCG).

Antisera Generation and Passive Transfer

Antisera directed against both eCG and hCG were generated in domestic cats as described in a previous study (Swanson et al., 1994b,c). Briefly, queens (n=6) were repeatedly stimulated at short intervals (49-57 days) using the standard eCG and hCG regimen and blood samples were collected from naive queens prior to the first eCG injection and then 18-25 days following the third stimulation. Sera were screened by ELISA for eCG-binding activity and sera from positive queens (n=4) (and naive sera from the same queens) were each pooled in equal volumes. Pooled sera was evaluated by ELISA for both eCG and hCG-binding activity.

Naive queens (n=10) were randomly assigned to one of two treatment groups (negative sera/control, n=5; positive sera/antisera, n=5) and stimulated with eCG/hCG. Following follicular aspiration at laparoscopy, queens were injected intravenously with either positive sera (1 ml) or negative sera (1 ml). Blood samples were collected prior to eCG treatment, at the initial laparoscopy, 2 hours post-sera transfer and at each subsequent laparoscopic procedure (5 and 15 days following follicular aspiration). Four queens in each treatment group were stimulated again with eCG/hCG at a 44- to 45-day interval and blood samples were collected prior to eCG, at laparoscopy and weekly for 4 weeks post-laparoscopy. All sera was stored frozen at -20°C until analyzed by ELISA (eCG-binding activity) and/or RIA (eCG and progesterone concentration).

Solid-phase ELISA

ELISA procedures were as previously described (Swanson et al., 1994b,c) with slight modification. Briefly, eCG and hCG (Sigma) were added to wells (500 ng protein/well) of microtiter plates and incubated, in succession, with serially-diluted (1:100-1:3200) serum samples, horseradish peroxidase (HRP)-conjugated, affinity purified rabbit anti-cat IgG (Zymed Laboratories, Inc., South San Francisco, CA) and *o*-

phenylenediamine solution (Sigma). The color reaction was stopped with H_2SO_4 and the optical density (OD) of wells was measured at 492 nm using an automated microplate reader. Each microtiter plate included serum samples from each treatment group and a negative serum reference, consisting of pooled naive sera (1:100 dilution) from all queens in the study. Mean OD values (± SD) were determined for the triplicate wells of the negative serum reference and OD values for serially diluted serum samples above the mean value for the plate-specific negative reference +3 SD (for all negative reference wells) were considered positive for the presence of gonadotropin-binding immunoglobulins. The end-point titer was the inverse (x 10^{-2}) of the highest dilution that was positive and samples that were negative at every dilution were assigned a titer of zero.

Progesterone RIA and Protein A Sepharose Immunoabsorbence

Serum samples from queens in control and antisera treatment groups were assayed for progesterone and eCG concentrations. Serum progesterone levels were determined by RIA (Thompson et al., 1983), following validation of the assay for cat serum. For validation, the RIA was shown to be specific by thin layer chromatography for progesterone in cat sera and parallelism of binding curves between the progesterone standard and serially diluted cat sera was demonstrated. Serum samples (100 µl) were assayed in duplicate using rabbit anti-progesterone antisera and ^{3}H-progesterone as the radiolabeled ligand and bound and unbound progesterone were separated by stripping with dextran-coated charcoal. All serum samples were evaluated in one assay, with an intra-assay coefficent of variation of 5% and an assay sensitivity of approximately 35 pg/ml.

Prior to RIA for eCG, cat IgG was absorbed from serum samples using Protein A Sepharose B (Zymed) gel columns. Serum samples (200 µl) were passed through columns composed of 4 cm beds of gel (containing ~1 ml swollen gel), buffered with a 0.1 M Na_2HPO_4 solution (pH 7.3), and the first fraction (500 µl) following the void volume (700 µl) was collected. Aliquots (100 µl) of the first fraction were assayed for eCG (as described above) and eCG concentrations were corrected for the original serum sample

volume. The first fraction typically contained ≥90% of unbound eCG. The binding capacity per ml of gel was 24 mg IgG so columns were regenerated with 0.1 M citric acid buffer (pH 3.5) after every three samples.

Statistical Analysis

The mean number (± SEM) of follicles and CL, the mean progesterone and eCG concentrations, the proportion of recovered oocytes classified as mature, the proportion of aspirated follicles forming CL and the proportion of queens with excessive secondary follicles were calculated for queens in control and antisera treatment groups, following the initial laparoscopy and subsequent laparoscopic procedures (if appropriate). Differences in means between groups were compared with a Student's *t*-test or paired *t*-test (Steel and Torrie, 1960) and proportions were evaluated using a Chi square analysis or Fisher exact test (Steel and Torrie, 1960).

Results

Rate of eCG Elimination

Following intramuscular injection of eCG 12 hours earlier, the mean (± SEM) serum eCG concentration was 108.1 ± 37.9 mIU/ml but individual values varied considerably between queens (Cat No. 53,165.6 mIU/ml; Cat No. 58, 122.1 mIU/ml; Cat No. 56, 36.7 mIU/ml). Elimination of eCG appeared linear for all queens between 12 and 84 hours post-injection, with an estimated elimination half-life of 24-48 hours for this time period (Figure 5). At laparoscopy, all queens exhibited multiple ovarian follicles (21, Cat No. 53; 15, Cat No. 58; 12, Cat No. 56; mean 16.0 ± 2.6) and serum eCG concentrations averaged 7.0 ± 3.3 mIU/ml (range 1.8-13.1 mIU/ml). At 2 days post-laparoscopy (or ~156 hours post-eCG injection), eCG was still detectable in sera obtained from all queens. However, cross-reactivity of the assay with residual hCG in serum samples may have slightly increased the latter values.

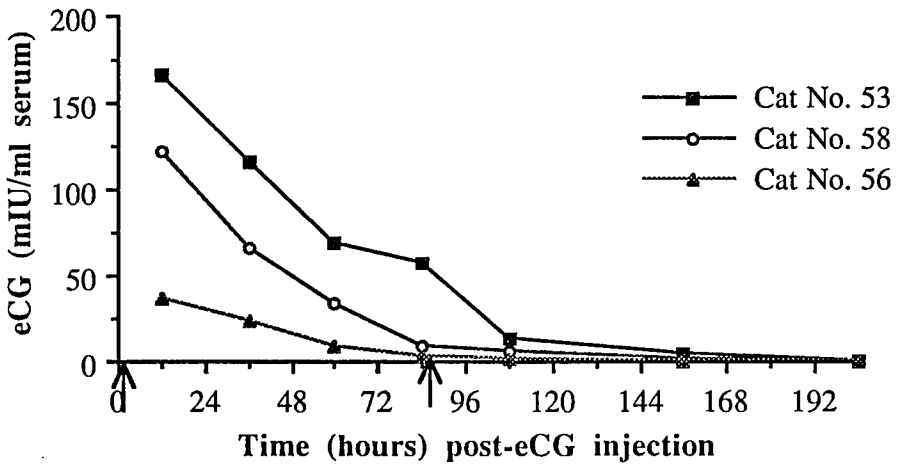

Figure 5. Serum concentrations of eCG (mIU/ml) in domestic cats (n=3) at specific time periods (12, 36 , 60 , 84 , 108, 156 and 204 hours) following intramuscular injection of 150 IU of eCG (first arrow). Queens were injected (i.m.) with 100 IU hCG (second arrow) at 84 hours and subjected to laparoscopy at 108 hours.

Laparoscopic Observations and Effect of Passive Transfer

At the initial laparoscopy, queens in the control (or negative sera) group had similar ($P<0.05$) mean numbers of observed follicles (26.2 ± 1.3) and CL (0.8 ± 0.8) to queens in the antisera treatment group (26.0 ± 3.1, 1.4 ± 0.9, respectively). After correcting follicle number for a greater number of recovered oocytes in individual queens, the mean follicle number for the control (26.6 ± 1.2) and antisera group (30.2 ± 5.6) did not differ ($P>0.05$) (Figure 6) and the proportion of recovered oocytes classified as mature was similar ($P>0.05$) between groups (control, 117/117 or 100%; antisera, 129/132 or 97.7%).

At 5 days post-follicular aspiration, both groups had high mean numbers of CL (control, 27.8 ± 2.9; antisera, 21.6 ± 4.4) and secondary follicles (control, 13.0 ± 3.3; antisera, 10.4 ± 5.2) but these values did not differ ($P>0.05$) between treatment groups (Figure 6). The proportion of queens with >5 ovarian follicles was not significantly different between groups, however, the tendency ($P=0.08$, Fisher) was for a greater proportion with control (5/5, 100%) versus antisera (2/5, 40%) groups. In addition, the proportion of aspirated follicles forming CL by Day 5 post-aspiration was greater ($P<0.05$) for the control (135/133, 102%) compared with the antisera (101/151, 66.9%) group.

At 15 days post-follicular aspiration, both groups had similar ($P>0.05$) mean numbers of CL (control 43.2 ± 8.7; antisera, 36.8 ± 5.3) and follicles (control, 2.0 ± 1.3; antisera, 5.4 ± 2.8) (Figure 6). Compared to the number of follicles and CL detected at follicular aspiration, secondary CL formation accounted for 15.8 ± 8.4 CL (range -2-47) in the control group and 5.2 ± 1.5 CL (range 2-10) in the antisera group and did not differ ($P>0.05$) between groups. Four queens in each treatment group were stimulated a second time with eCG/hCG (at a 44- to 45-day interval) and laparoscopy revealed a similar ($P>0.05$) mean number of ovarian follicles (control, 24.3 ± 6.5; antisera, 21.5 ± 8.3) and proportion of recovered mature oocytes (control, 103/103 or 100%; antisera, 63/63 or 100%) between groups.

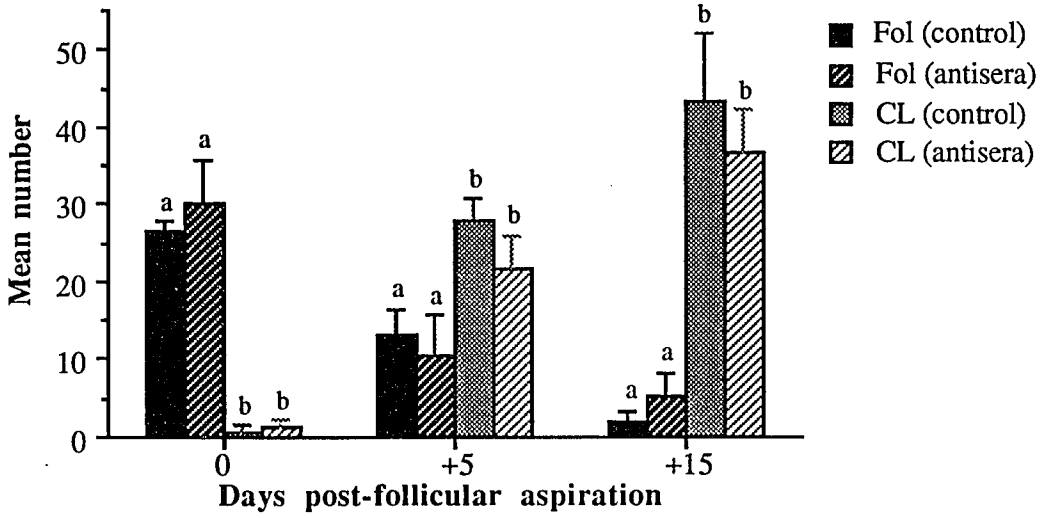

Figure 6. Mean number (± SEM) of follicles and CL observed at laparoscopy in eCG/hCG-stimulated queens at specific days (0, +5, +15) post-follicular aspiration. Queens were infused on Day 0 with pooled sera that was either negative (control) or positive (antisera) for eCG/hCG-binding activity. Within time periods, values with different superscripts are significantly different ($P<0.05$).

Progesterone and eCG Concentrations

At initial laparoscopy, mean serum progesterone concentrations were slightly but similarly elevated (P>0.05) for queens in control (3.5 ± 1.6 ng/ml) and antisera (3.8 ± 1.2 ng/ml) groups (Figure 7a). One queen in the control group and two queens in the antisera group (Figure 7b) each exhibited premature ovulation of 3-4 follicles, likely accounting for the early progesterone elevation. At 5 days post-follicular aspiration, mean serum progesterone concentrations had increased significantly (P<0.05) but similarly (P>0.05) for both treatment groups (45.7 ± 5.3 ng/ml, control; 46.0 ± 8.9 ng/ml, antisera) (Figure 7a). When adjusted for the number of CL/queen, mean serum progesterone/CL did not differ (P>0.05) between groups. (Figure 7b) At 15 days post-aspiration, mean serum progesterone concentrations and serum progesterone/CL did not differ (P>0.05) for queens in control (83.9 ± 25.2 ng/ml; 1.9 ± 0.3 ng/ml/CL) and antisera (92.8 ± 20.7 ng/ml; 2.4 ± 0.3 ng/ml/CL) groups.

At 12 hours post-eCG injection, mean eCG serum concentrations for control (88.3 ± 22.5 mIU/ml) and antisera (59.9 ± 12.2 mIU/ml) groups did not differ (P>0.05) from each other nor from mean eCG levels (108.1 ± 37.9 mIU/ml) reported in the eCG elimination study. Similarly, at laparoscopy, serum concentrations of eCG did not differ (P>0.05) for control sera (5.0 ± 1.5 mIU/ml), antisera (8.4 ± 4.4 mIU/ml) or eCG elimination (7.0 ± 3.3 mIU/ml) treatment groups. Following infusion of negative or positive sera, eCG concentrations were similar (P>0.05) for both treatment groups (2.8 ± 0.9 mIU/ml, control; 4.4 ± 3.5 mIU/ml, antisera) and did not decrease significantly (P>0.05) for either group relative to pre-infusion eCG levels. By 5 days post-infusion, eCG was undetectable in sera from queens in either treatment group.

Humoral Immune Responses

For sera infused in the passive transfer study, ELISA results indicated that the pooled sera from immunorefractory queens was positive for eCG-binding at a titer of 4 (1:400 dilution) and for hCG-binding at a titer of 1 (1:100 dilution). Pooled naive sera

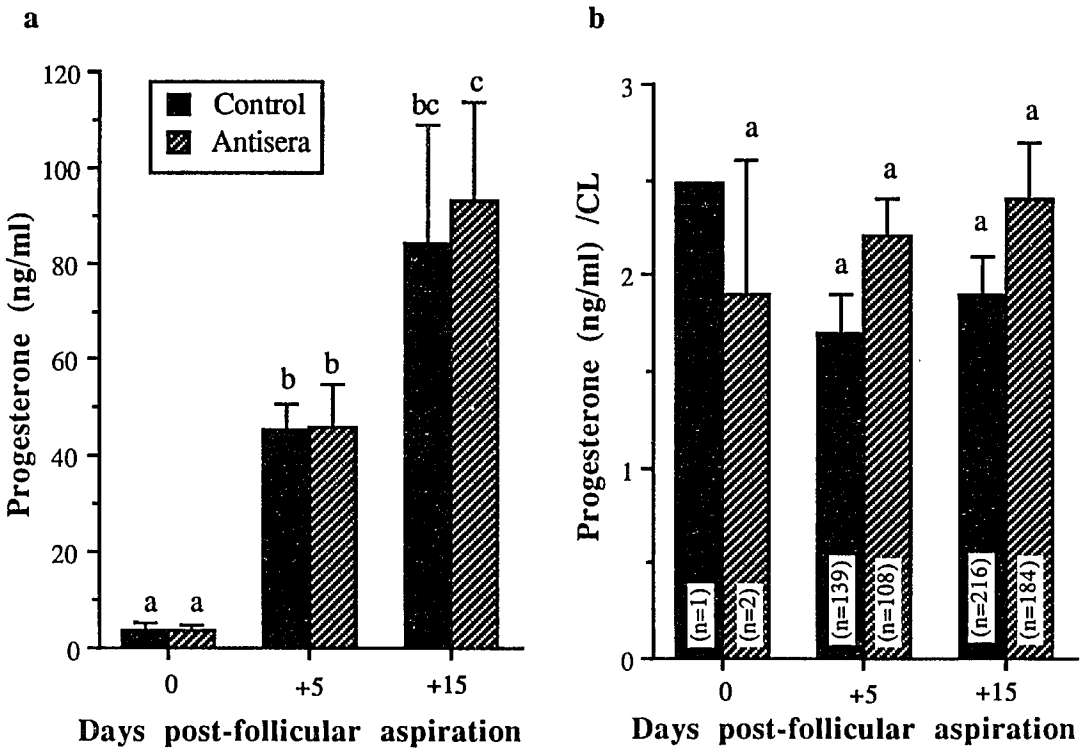

Figure 7. (a) Serum progesterone concentration (ng/ml) and (b) serum progesterone concentration per CL (ng/ml/CL) for eCG/hCG-stimulated queens at specific days (0, +5 and +15) post-follicular aspiration. On Day 0, queens were infused with pooled sera that was either negative (control) or positive (antisera) for eCG/hCG-binding activity. Numbers in parentheses within columns represent CL per treatment group. Within graphs, values with different superscripts are significantly different ($P<0.05$).

from these same four queens were negative for both eCG and hCG-binding (titers of 0). At 2 hours following passive transfer of negative and positive sera, anti-eCG serum titers in infused queens were unchanged for all queens in the control group and four of five queens in the antisera group. One antisera queen (Cat No. 66) exhibited a one-dilution increase in titer following sera infusion.

Prior to the second stimulation with eCG/hCG, anti-eCG serum titers were negative in three of four queens in each treatment group (Figure 8). Following eCG injection, serum titers increased at least one-dilution within 2 weeks in three antisera and four control treatment queens and peak serum titers were 4 or less in all queens except one control queen (Cat No. 67, Figure 8a). Serum titers remained elevated at least 4 weeks for two queens in each treatment group (Figure 8a and 8d).

Discussion

In the present study, serum concentrations of eCG were highly variable in domestic cats (of similar body weight) following intramuscular injection, but all cats possessed multiple (≥12) ovarian follicles at laparoscopy. Similar variability of eCG concentrations following intramuscular injection have been reported in cattle (Yadav et al., 1986; Bevers and Dieleman, 1987) and sheep (Meinecke-Tillmann et al., 1987), with contradictory effects on ovarian responses. In cattle, one study (Bevers and Dieleman, 1987) reported no apparent correlation but another study (Yadav et al., 1986) indicated that slower rates of eCG absorption produced greater numbers of large follicles. In sheep (Meinecke-Tillmann et al., 1987), lower eCG serum concentrations 24-48 hours post-injection have been associated with decreased numbers of CL . Most likely, this initial variability of eCG concentrations in cats (and other species) was caused by individual variation in eCG absorbance and distribution from intramuscular sites.

In cats, the elimination half-life of eCG (for the initial 72 hour period post-injection) was estimated at 24-48 hours, which is intermediate to the pharmacological elimination

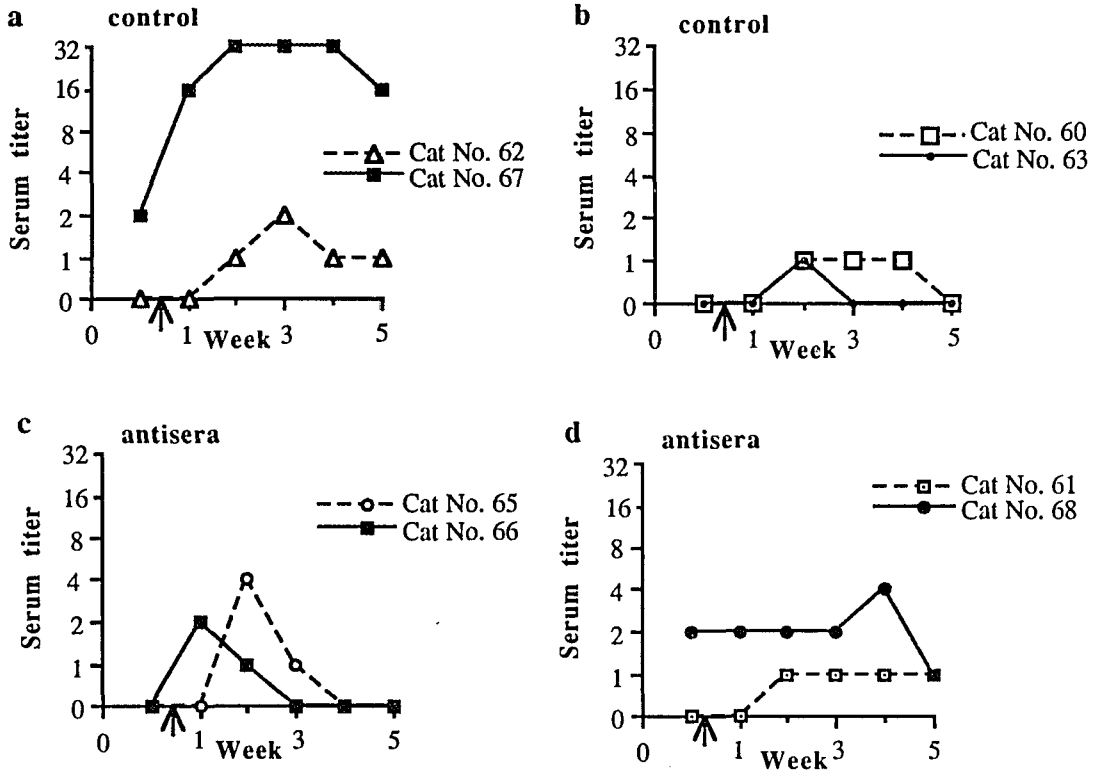

Figure 8. Secondary immune responses (serial eCG-binding immunoglobulin titers (x 10^{-2})) of eCG/hCG-stimulated queens previously infused with pooled sera that was either (a, b) negative (control) or (c, d) positive (antisera) for eCG/hCG-binding activity. Arrows indicate the second eCG injection (44- to 45-day interval) with serum titers at laparoscopy represented by the next data point.

half-life of eCG determined for cattle (40-51 hours; Menzer and Schams, 1979) and sheep (21 hours; McIntosh et al., 1975). Estimation of eCG elimination (after 84 hours) and persistence in circulation was confounded by a low level of cross-reactivity of the eCG RIA with hCG. The elimination half-life of hCG in cats also is unknown but, generally, hCG is less persistent in circulation than eCG, with the circulatory half life in other species ranging from 48 minutes (rat; Kalyan et al., 1982) to 24 hours (primate; Stouffer et al., 1986).

In the present study, the apparent changes in slope of the eCG elimination curves, following hCG injection, may reflect a biphasic elimination rate, as reported in cattle (Menzer and Schams, 1979) and rats (Aggarwal and Papkoff, 1985) or be an artifact of hCG cross-reactivity. Similarly, the prolonged persistence of eCG for 6-7 days post-injection may be slightly exaggerated by hCG cross-reactivity. However, based on the inital mean eCG serum concentration, the estimated rate of elimination and the sensitivity of the RIA, eCG should be detectable in serum for at least five times the half-life or 5-10 days post-injection in cats. These results indicate that low concentrations of eCG still may be present in circulation following follicular aspiration, possibly contributing to secondary follicular growth or luteinization. With the use of combination gonadotropin regimens, however, the formation of secondary ovarian structures in cats might be attributable to the persistence of eCG, hCG or both.

Following eCG/hCG stimulation and follicular aspiration, queens in the control (negative sera) group demonstrated rapid development of CL and a significant increase in serum progesterone levels by 5 days post-aspiration. While the number of observed CL closely corresponded to the number of follicles aspirated 5 days earlier, queens also exhibited a high number of ancillary ovarian follicles. Because the dynamic nature of estradiol-17ß secretion requires frequent blood sampling to obtain accurate measurements (Saumande, 1980; Wildt et al., 1981), the functionality of these ancillary follicles could not be determined in this study. However, limited results (Goodrowe et al., 1988a; Donoghue et al., 1992b) indicate that estradiol-17ß concentrations remain elevated for several days in

cats following follicular aspiration, suggesting continued follicular development and function.

The laparoscopic observations are consistent with previous studies (Goodrowe et al., 1988a; Donoghue et al., 1992b), which showed that essentially all aspirated follicles rapidly luteinize and form functional CL within a few days post-aspiration. These earlier studies also indicated that, when examined at laparoscopy 7 days post-follicular aspiration, eCG/hCG-stimulated queens either had an excessive number of ancillary ovarian follicles (Donoghue et al., 1992b) or secondary CL (Goodrowe et al., 1988a). While identical gonadotropin dosages were used in each study, the intervals between eCG and hCG injections were slightly shorter (72 or 80 hours) in the study by Goodrowe et al. (1988a). The observation of secondary CL instead of ancillary follicles may reflect altered follicular dynamics and a more rapid synergistic action of eCG and hCG on ancillary follicular development and luteinization compared with the longer eCG/hCG intervals. Additionally, the formation of ancillary follicles and secondary CL does not appear to be a consequence of follicular aspiration, since queens stimulated with eCG/hCG and allowed to ovulate in laparoscopic AI procedures also exhibit multiple secondary CL when examined several days post-AI (Howard et al., 1992a).

In the present study, almost all ancillary ovarian follicles observed at 5 days post-aspiration became CL within the next 10 days and few additional follicles were formed. Because serum eCG and hCG concentrations were likely minimal by 5 days post-aspiration and queens typically require relatively high dosages (50-75 IU) of hCG to induce ovulation following eCG treatment (Howard et al., 1992a; Hamner et al., 1970), these follicles possibly luteinized without ovulation. Luteinization of ancillary follicles has been reported previously in cattle (Dieleman et al., 1989) and guinea pigs (Rawson et al., 1979) following eCG or eCG/hCG treatment, respectively. The further elevation in serum progesterone levels between 5 and 15 days post-aspiration is consistent with secondary CL

formation (Goodrowe et al., 1988a) as well as increased progesterone synthesis by primary CL (Pappe et al., 1975; Wildt et al., 1981).

The infusion of gonadotropin-neutralizing antisera, in an attempt to decrease ancillary follicle and secondary CL formation, was largely unsuccessful. While administration of antisera apparently slowed CL formation (at laparoscopy five days post-aspiration) and tended to reduce the number of queens with excessive ancillary follicles, the mean number of follicles and secondary CL and the mean serum progesterone concentrations were similar to that of control queens. In addition, secondary humoral immune responses did not differ qualitatively from control queens, as both groups had similar latent periods, peak elevations in anti-eCG titers and duration of elevated titers.

The ineffectiveness of antisera treatment may have several potential causes, including inadequate antisera treatment volume and/or bioactivity or improper timing of infusion. Pooled antisera exhibited a high titer of eCG-binding immunobglobulins and a slightly lower titer for hCG-binding, as indicated by ELISA, and similar pooled antisera previously had been shown to neutralize eCG/hCG bioactivity in mouse ovarian stimulation assays (Swanson et al., 1994b). In this previous study (Swanson et al., 1994b), it was demonstrated that an antisera volume of 50 µl was effective in attenuating the biological activity of 5 IU of eCG. Although anti-eCG serum titers did not increase in queens immediately following antisera infusion, antisera binding capacity (at ~1:200 antisera:blood volume dilution) should have been adequate to neutralize the relatively low concentrations of circulating eCG. It is possible that eCG, but not hCG, was neutralized and that hCG was the primary cause of secondary follicular formation. Although eCG has both FSH and LH-like activity (Licht et al., 1979) and hCG is generally considered LH-like in function, hCG also may exhibit some folliculogenic activity in cats (Goodrowe and Wildt, 1987).

Alternately, volume and activity of antisera may have been adequate but infusion was too late to prevent ancillary follicular development. Because antisera was cross-reactive for both eCG and hCG, infusion was delayed until after folliclular aspiration to

avoid disruption of final intra-follicular oocyte maturation. With a more specific antisera (or a monoclonal antibody; Dieleman et al., 1993) for eCG, eCG neutralization might be initiated shortly after the hCG injection. In eCG-stimulated cattle, anti-eCG immunoglobulins are most effective in improving embryo yield and reducing formation of post-ovulatory follicles when administered 6 and 18 hours after the endogenous LH surge (Dieleman and Bevers; 1987; Bevers et al., 1993), if given earlier, follicular dynamics are altered (Dieleman et al., 1993). Because cats are induced ovulators, close timing of anti-eCG therapy relative to the LH surge (i.e., hCG injection) is less problematic than in cattle (Dieleman et al., 1993), but the effect of earlier eCG neutralization on the follicular microenvironment and resulting oocyte quality still must be a concern.

Even with slightly earlier administration of specific anti-eCG immunoglobulins, the benefits of this passive immunization on secondary humoral immune responses probably would continue to be minimal or nonexistent. Repeated stimulation of queens with eCG and hCG may produce high anti-eCG/hCG serum titers and cause an immunologically-mediated decrease in ovarian responsiveness (Swanson et al., 1994b,c), with the interval between successive stimulations and the magnitude of the secondary immune response of critical importance (Swanson et al., 1994c). With the standard eCG/hCG regimen and the neutralization of eCG shortly after follicular aspiration (or possibly hCG administration), queens are still exposed to the immunostimulatory effects of eCG for at least three to four days post-injection. In addition, hCG may contribute to the formation of gonadotropin-specific immunoglobulins or immunoglobulins that are cross-reactive with eCG (Swanson et al., 1994b). Under these conditions, neutralization of eCG following primary exposure is unlikely to diminish the secondary humoral immune responses observed with subsequent stimulations.

CHAPTER V

RESISTANCE OF THE DEVELOPMENTAL BLOCK IN *IN VITRO* FERTILIZED DOMESTIC CAT EMBRYOS TO TEMPORAL VARIATIONS IN CULTURE CONDITIONS

Introduction

In both domestic and nondomestic felids, procedures for *in vitro* fertilization (IVF) of *in vivo* matured oocytes have provided an efficient mechanism for the generation of multiple embryos for use in pre-implantation developmental studies (Goodrowe et al., 1988a; Goodrowe et al., 1989; Miller et al., 1990; Johnston et al., 1991a,b; Donoghue et al., 1992b; Pope et al., 1993a). In addition, the culture and subsequent transfer of IVF embryos to synchronized recipients has allowed the production of offspring in both domestic (Goodrowe et al., 1988a; Pope et al, 1993a; Swanson and Godke, 1994a) and nondomestic cat species (Donoghue et al, 1990; Pope et al., 1989b), and these procedures exhibit great promise for the conservation of endangered cat species (Wildt, 1990; Wildt et al., 1992b). However, following IVF and embryo transfer (ET), embryo survival and/or pregnancy rates to date have been exceptionally low. While an unsuitable maternal environment in embryo recipients may be one potential cause for this low ET efficiency (Swanson et al., 1994a,d), additional factors, such as compromised developmental competence of IVF-derived embryos, may be at least partially responsible.

IVF-derived cat embryos will develop readily *in vitro* to the morula stage under a variety of culture conditions but most morulae will fail to continue development into blastocysts (Johnston et al., 1991a,b; Roth et al., 1994a). This morula-to-blastocyst block has proven resistant to alterations of protein source (Johnston et al., 1992a), media complexity (Johnston et al., 1992a), temperature and gas atmosphere (Johnston et al., 1991b) and, most recently, to tissue co-culture techniques (Roth et al., 1994a). Because these embryos consistently fail to form blastocysts in culture, questions are raised about the suitability of the culture systems as well as the biological competence of most IVF-derived embryos. The possibility for deficiencies with either factor was supported by a recent

study (Roth et al., 1994b) that suggested that IVF-derived cat embryos exhibit compromised development *in vitro* relative to *in vivo*-generated embryos but also that culture duration has a inverse negative effect on blastocyst formation by *in vivo*-generated embryos.

In other species, developmental blocks have responded to simple alterations in energy substrates and other media constituents (Kane and Foote, 1970; Schini and Bavister, 1988; Chatot et al., 1989) or to embryo co-culture with oviductal tissue (Gandolfi and Moor, 1987; Rexroad and Powell, 1988; Eyestone and First, 1989; White et al., 1989; Prichard et al., 1992). However, increasingly, evidence suggests that embryo developmental requirements are not static and that the composition of a single medium or culture system may be inadequate for all stages of embryo development (Chatot et al., 1989; Gardner and Leese, 1990; Brown and Whittingham, 1992). In the present study, the effects of different energy substrates (glucose and glutamine), media formulation (simple and complex) and tissue co-culture on the *in vitro* development of IVF-derived cat embryos were investigated. In addition, the effects of temporal variations in these different culture conditions were examined with particular attention given to the potential impact upon the morula-to-blastocyst developmental block.

Materials and Methods

Experimental Animals

Random source, adult female domestic cats (n=28) were conditioned and housed singly or in pairs in stainless steel cages as previously described (Swanson et al., 1994a,b). A single proven sperm cell donor was maintained in a separate cage under similar environmental conditions. All cats were provided a commercial cat diet (Science Diet, Hill's Pet Products, Topeka, KS) and water *ad libitum*.

Gonadotropin Stimulation, Laparoscopy and Oocyte Recovery

Exogenous gonadotropin treatment and laparoscopy were as previously described (Swanson et al., 1994a,b), based on slight modification of earlier studies (Wildt et al., 1977; Goodrowe et al., 1988a; Johnston et al., 1991a,b). Briefly, queens were administered (i.m.) equine chorionic gonadotropin (eCG; 150 IU; Sigma Chemical Co., St. Louis, MO), followed 84 hours later with human chorionic gonadotropin (hCG; 100 IU; i.m.; Sigma), and anesthetized 24 to 27 hours after hCG treatment to evaluate ovarian responses via laparoscopy. Mature ovarian follicles (≥2 mm) were aspirated trans-abdominally into 10 ml vials containing modified Kreb's Ringer medium (mKrb) (Toyoda and Chang, 1974) supplemented with 0.4% bovine serum albumin (BSA; Fraction-V; Sigma) and 40 units of heparin per ml but modified to exclude glucose (Study I and II) or to substitute glutamine for glucose (Study III). Prior to processing, recovered oocytes were maintained 1- to 4-hours post-collection in aspiration vials equilibrated at 38°C in 5% CO_2 in air.

Aspiration vials were emptied into 60 mm plastic Petri dishes and recovered oocytes were evaluated and assessed for maturational status, based on previously described criteria (Johnston et al., 1991a,b). All mature oocytes were combined in 3 ml of mKrb (without heparin but modified for Study I, II and III as above), washed through three microdrops of the appropriately modified mKrb and randomly assigned to treatment groups for IVF. Oocytes were transferred to 100 µl drops (5-10 oocytes/drop) of fertilization medium under oil and then equilibrated in the incubator for 1 hour prior to insemination.

Semen Collection and Processing

A single male was used for all IVF procedures as previously reported (Johnston et al., 1991a,b) with semen collection by electroejaculation as previously described (Wildt et al., 1983). Recovered fresh semen was placed into a 1.5 ml centrifuge vial, diluted in 100 µl of mKrb (either without glucose for Study I and II or with glutamine substituted for glucose for Study III) and centrifuged for 8 min at 300 x g. The supernatant was removed,

the sperm pellet was overlaid with 50 µl mKrb (modified as above) and the sperm cells were incubated at 38°C (in 5% CO_2 in air) for 30 minutes to allow sperm swim-up (Howard et al., 1990). The swim-up layer was carefully removed, transferred to a second centrifuge tube and evaluated for sperm concentration, percent motility and rate of progressive motility (Howard et al., 1990). The sperm cells were diluted with additional mKrb (modified as above) to a concentration of 2.5 x 10^6 motile sperm cells per ml and used immediately for IVF.

***In Vitro* Fertilization**

Oocytes in each fertilization drop were inseminated with 10 µl of diluted sperm (2.5 x 10^4 motile sperm), co-incubated for 6 hours and then washed through three microdrops of the culture medium. Oocytes were transferred to four-well tissue culture plates (Nunclon, Copenhagen, Denmark), containing 500 µl of equilibrated medium overlaid with 400 µl washed mineral oil, and cultured *in vitro* according to protocols for Study I, II and III. Embryos were evaluated every 24 hours for morphological development, with embryos developing to >16 cells classified as morulae and embryos forming a blastocoelic cavity as blastocysts. After 144 hours of culture, embryos classified as ≥8 cells were stained with DNA-specific Hoescht stain 33342 (Pursel et al., 1985) to verify developmental status and to determine the number of cell nuclei per embryo.

Study I - Effect of Energy Source (Glucose and Glutamine)

Oocytes were collected in mKrb (without glucose or glutamine) and randomly assigned to fertilization drops containing standard mKrb (with glucose) or mKrb without glucose but with glutamine (1 µM per ml) (Figure 9). After a 6 hour fertilization period and an additional 18 hours of culture in 1 ml culture wells, oocytes were stripped of cumulus cells by gentle pipetting and transferred to culture wells containing fresh medium. At 48 hours post-insemination, oocytes were assessed for cleavage to ≥2 cells as an indication of fertilization and embryos were transferred to fresh four-well culture plates. Each 24 hours, after microscopic evaluation, embryos were transferred to wells containing

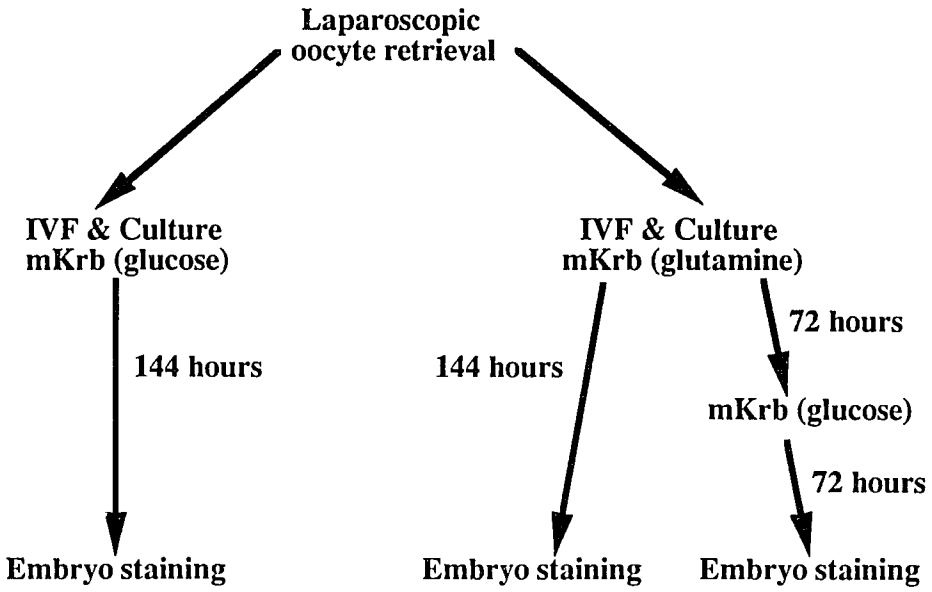

Figure 9. Flow diagram of culture treatments for Study I: Effect of energy source on development of IVF-derived cat embryos.

fresh, equilibrated medium. Embryos were cultured either A) in mKrb (glucose) or B) in mKrb (glutamine) for the entire 144 h culture period or C) maintained in mKrb (glutamine) for the first 72 hours and then transferred to mKrb (glucose) for the final 72 hours. The study was conducted in five replicates with the medium (mKrb) prepared from stock chemicals (Sigma) on the day prior to oocyte recovery. For different mKrb formulations, osmolarity was equalized (~280 mOsm) by addition of NaCl, when needed. The culture medium was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and filtered (0.22 μm; Acrodisc, Gelman, Ann Arbor, MI) prior to use.

Study II - Effect of Energy Source and Media Complexity

Oocytes were collected in mKrb (without glucose or glutamine) and randomly assigned to fertilization drops of mKrb (glucose), mKrb (glutamine), or Ham's F-10 (HF-10) culture medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) (Figure 10). After fertilization, embryos were transferred to four-well plates containing the respective media and, after an additional 18 hours of culture, oocytes were stripped of cumulus cells and transferred to new culture wells. Embryos were cultured either A) in HF-10 for the entire 144 hours or B) in mKrb (glucose) or C) mKrb (glutamine) for the first 72 hours and then transferred to HF-10 for the final 72 hours. The study was conducted in two replicates with the media prepared (on the day prior to oocyte recovery) either from commercially-formulated liquid medium (HF-10), freshly supplemented with glutamine (1 μM per ml) and FBS, or from stock chemicals (mKrb), adjusted with NaCL to equal osmolarity. All medium was supplemented with antibiotics and filtered prior to use.

Study III - Effect of Energy Source, Media Complexity and Oviductal Cell Co-Culture

To obtain oviduct cells, reproductive tracts were recovered from adult queens at ovariohysterectomy and the oviducts were isolated and ligated. Oviducts were infused with 0.25% trypsin (Sigma) in HF-10, incubated at 38°C for 30 min and flushed with HF-10

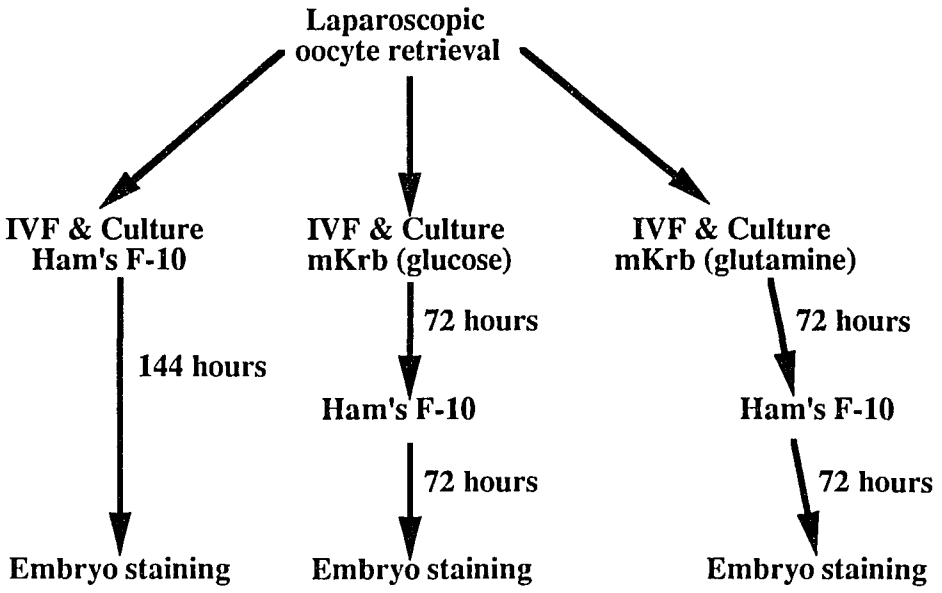

Figure 10. Flow diagram of culture treatments for Study II: Effect of temporal variations in energy source and media complexity on development of IVF-derived cat embryos.

(with 10% FBS). The recovered cells were diluted in 10 ml HF-10, centrifuged for 10 minutes at 300 x g and each cell pellet resuspended in 4 ml fresh HF-10. Resuspended cells were placed in 50 ml plastic tissue culture flasks for incubation at 38°C in 5% CO_2 in air. After 5 to 7 days of culture, nonadherent cells were rinsed from the flasks and the remaining cellular monolayer was freed from the plastic substrate following incubation with 0.25% trypsin (in HF-10). Following washing and centrifugation, cells were resuspended in 1 ml of cryopreservation solution (HF-10 with 10% FBS and 7% dimethylsulfoxide) and frozen in liquid nitrogen.

To establish monolayers for co-culture, cells were thawed and incrementally re-equilibrated in HF-10. Four days prior to co-culture, wells of the culture plates were seeded with frozen-thawed oviduct cells (7.5 - 10.0 x 10^4 cells/well) in 500 µl HF-10 (with 10% FBS) and overlaid with 400 µl washed oil. Each 1-ml well was rinsed and replenished with fresh medium 24 h prior to the onset of co-culture. The study was conducted in two replicates with the medium prepared as outlined for Study II.

Oocytes were collected in mKrb (glutamine), randomly assigned to treatment groups and inseminated in drops of mKrb (glutamine) (Figure 11). After 6 hours of co-culture, embryos were stripped of cumulus cells and transferred to culture wells of medium alone or containing a monolayer of cat oviduct cells. At 24-hour intervals, embryos were evaluated and half (250 µl) of the medium in each well was removed and replaced with fresh medium. Embryos were cultured either A) in HF-10 (with 10% FBS) alone or B) on oviductal cell monolayers (in HF-10) for the entire 144 hours or C) and D) in mKrb (glutamine) for the first 72 hours with a transfer to either C) HF-10 or D) oviductal cell monolayers for the final 72 hours.

Statistical Analysis

Within each study, fertilization percentages and the proportion of morulae, blastocysts and blastocysts/morulae were compared for each treatment group using Chi square analysis (Steel and Torrie, 1960). The mean number (± SEM) of cells per embryo

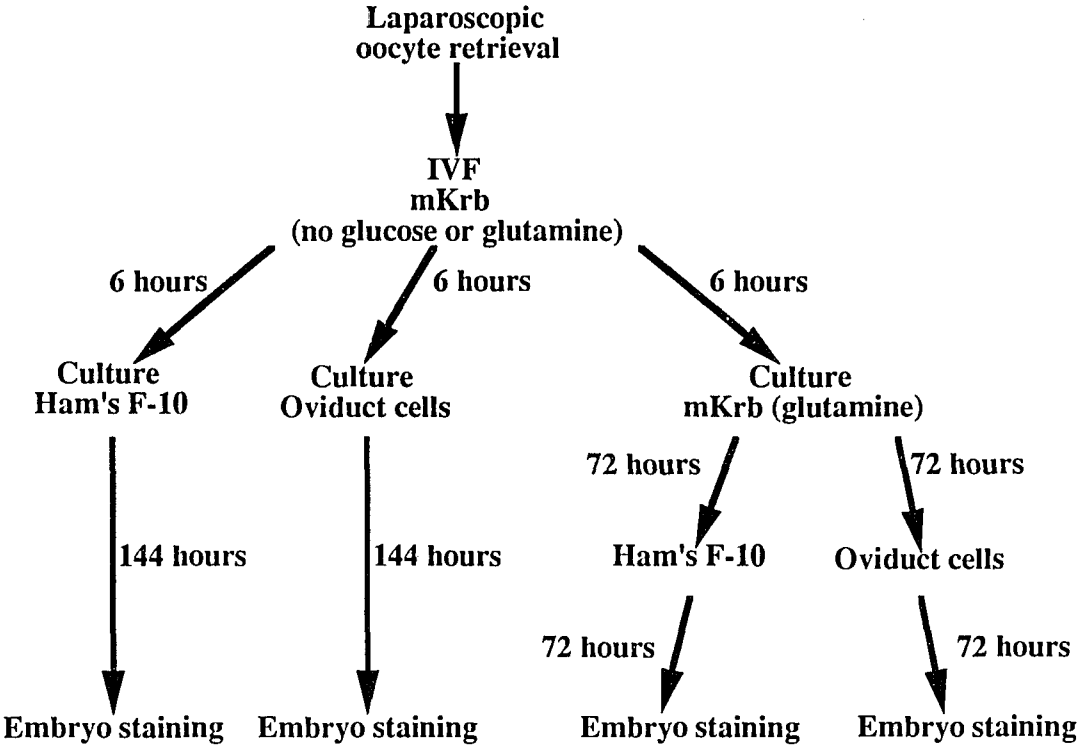

Figure 11. Flow diagram of culture treatments for Study III: Effect of temporal variations in energy source, media complexity and oviductal cell co-culture on development of IVF-derived cat embryos.

was determined for each treatment group and evaluated using analysis of variance. Differences between groups were assessed with a Tukey's means comparison test (SAS, 1984).

Results

Study I

The overall fertilization rate (cleavage to ≥2 cells) in mKrb was 80.3% (171/213) with similar (P>0.05) cleavage percentages for mKrb (glucose) (58/75; 77.3%) and mKrb (glutamine) (113/138; 81.8%). In each treatment group, a similar (P>0.05) percentage (range 77.6 - 89.3%) of 2-cell embryos developed to the morula stage with no difference (P>0.05) in the percentage (range 2-5%) developing to blastocysts (Table 6). Furthermore, the number of cells per embryo did not differ (P>0.05) between treatment groups (Table 6). The percentage of morulae forming blastocysts was universally low for all treatments, ranging from 2.2% to 6.0%, and was not different (P>0.05) among groups.

Study II

The overall fertilization rate in mKrb and HF-10 was 63.5% (122/192), with cleavage percentage (42.6%; 29/68) for HF-10 lower (P<0.05) than for mKrb (glucose) (73.4%; 47/64) or mKrb (glutamine) (76.7%; 46/60) treatment groups. The development of 2-cell embryos to morulae was similar (P>0.05) for all treatment groups (range 59.6 - 79.3%) but a greater percentage of blastocysts (P<0.05) were formed in mKrb (glutamine)/HF-10 treatment group (17.4%) than in mKrb (glucose)/HF-10 (2.1%) treatment group (Table 7). Blastocyst percentage for HF-10 alone (6.9%) was not different (P>0.05) from the other two groups (Table 7). Similarly, a greater (P<0.05) percentage of morulae formed blastocysts in mKrb (glutamine)/HF-10 (22.9%; 8/35) than in mKrb (glucose)/HF-10 (3.6%; 1/28) but morula-to-blastocyst percentage in HF10 alone (8.7%; 2/23) was not different (P>0.05).

Table 6. Effect of energy source on development of IVF-derived domestic cat embryos during *in vitro* culture

Culture treatment[a]	No. of 2-cell embryos	No. of embryos developing to morulae (%)[b]	No. of embryos developing to blastocysts (%)	Cell no. per embryo[c]
A mKrb (Glut)	58	45 (77.6%)	1 (2%)	27.6 ± 1.3
B mKrb (Glut)	57	45 (78.9%)	1 (2%)	29.1 ± 1.4
C mKrb (Glut)/ mKrb(Gluc)	56	50 (89.3%)	3 (5%)	31.9 ± 1.3

[a] Embryos (2-cell) were cultured either in (A) mKrb (glucose) or (B) mKrb (glutamine) for 144 hours or in (C) mKrb (glutamine) for the first 72 hours followed by mKrb (glucose) for the last 72 hours.

[b] Embryos >16 cells.

[c] Embryos ≥8 cells were stained with Hoescht 33342 and mean cell number (± SEM) determined for each treatment group.

Table 7. Effect of temporal variations in energy source and media complexity on development of IVF-derived domestic cat embryos during *in vitro* culture

Culture treatment[a]	No. of 2-cell embryos	No. of embryos developing to morulae (%)[b]	No. of embryos developing to blastocysts (%)	Cell no. per embryo[c]
A HF-10	29	23 (79.3%)	2 (6.9%)[de]	25.2 ± 1.6[de]
B mKrb (Gluc)/ HF-10	47	28 (59.6%)	1 (2.1%)[d]	23.4 ± 1.5[d]
C mKrb (Glut)/ HF-10	46	35 (76.1%)	8 (17.4%)[e]	30.2 ± 1.9[e]

[a] Embryos (2-cell) were cultured either (A) in HF-10 alone for 144 hours or (B) in mKrb (glucose) or (C) mKrb (glutamine) for the first 72 hours followed by HF-10 for the last 72 hours.

[b] Embryos >16 cells.

[c] Embryos ≥8 cells were stained with Hoescht 33342 and mean cell number (± SEM) determined for each treatment group.

[d,e] Values within columns with different superscripts are significantly different ($P<0.05$).

Study III

Overall fertilization rate for all treatments was 79.1% (167/211) but cleavage percentage was lower ($P<0.05$) for oviduct cells (64.2%; 34/53) than for HF-10 alone (84.6%; 44/52), mKrb (glutamine)/HF-10 (83.0%; 44/53) and mKrb (glutamine)/oviduct cells (84.9%; 45/53). Embryo development to the morula stage was greater ($P<0.05$) for HF-10 alone (72.7%) and mKrb (glutamine)/oviduct cells (73.3%) than for oviduct cells alone (44.1%) but was not different ($P>0.05$) from mKrb (glutamine)/HF-10 (63.6%). However, more ($P<0.05$) embryos developed to blastocyst stage in mKrb (glutamine)/HF-10 (11.4%) than on oviduct cells (0%), with the remaining two treatments intermediate and not different ($P>0.05$) (Table 8). With the mKrb (glutamine)/HF-10 treatment, 17.9% (5/28) of morulae developed to the blastocyst stage but this value was not different from that observed with HF-10 alone (3.7%; 1/32), oviduct cells alone (0%; 0/15) or mKrb (glutamine)/oviduct cells (6.1%; 2/33).

Discussion

The results of these studies have confirmed previous findings that IVF cat embryos fertilize readily and develop to the morula stage under a variety of culture conditions but also that the morula-to-blastocyst transition *in vitro* remains largely resistant to these variations. While temporal variations in energy sources, media complexity and tissue co-culture revealed minor differences in embryo developmental requirements during different periods of culture, the specific culture conditions required to resolve the *in vitro* developmental block were not determined in this series of studies. As a consequence, the biological competence of most IVF-derived cat embryos still must be considered suspect.

In the first study, substituting glutamine for glucose in mKrb was insufficient in promoting greater blastocyst formation or increasing embryo cell number; however, the results did demonstrate that fertilization and development to morulae were not affected by this alteration in energy source. A previous study (Johnston et al., 1991a) indicated that cat

Table 8. Effect of temporal variations in energy source, media complexity and oviduct cell co-culture on development of IVF-derived domestic cat embryos during *in vitro* culture

Culture treatment[a]	No. of 2-cell embryos	No. of embryos developing to morulae (%)[b]	No. of embryos developing to blastocysts (%)	Cell no. per embryo[c]
A HF-10	44	32 (72.7%)[d]	1 (2.3%)[de]	34.9 ± 2.7
B Oviduct cell co-culture	34	15 (44.1%)[e]	0 (0%)[d]	25.4 ± 3.3
C mKrb (Glut)/ HF-10	44	28 (63.6%)[de]	5 (11.4%)[e]	36.1 ± 4.3
D mKrb (Glut)/ Co-culture	45	33 (73.3%)[d]	2 (4.4%)[de]	36.1 ± 3.0

[a] Embryos (2-cell) were cultured either in (A) HF-10 or on (B) oviductal cell monolayers (in HF-10) for 144 hours or in (C) and (D) mKrb (glutamine) for the first 72 hours followed by (C) HF-10 or (D) oviductal cell monolayers (in HF-10) for the last 72 hours.

[b] Embryos >16 cells.

[c] Embryos ≥8 cells were stained with Hoescht 33342 and mean cell number (± SEM) determined for each treatment group.

[d,e] Values within columns with different superscripts are significantly different ($P<0.05$).

embryos develop equally well to the morula stage in standard mKrb (Toyoda and Chang, 1974) and another simple medium, TALP (Bavister and Yanagimachi, 1977) (modified to exclude glucose and phosphate), but that blastocyst formation was very low (3-6% of embryos) in both media. The present study investigated the possibility that glucose was detrimental to later blastocyst formation when present during the early culture period but was required for later blastocyst development, and that glutamine was the preferable energy source (to ~ 9- to 16-cell stage) during the first 72 hours of embryo culture.

In other species, such as mice (Chatot et al., 1989), hamsters (Schini and Bavister, 1988) and cattle (Ellington et al., 1990; Pinyopummintr and Bavister, 1991), early *in vitro* developmental blocks (<16 cell stage) and/or later blastocyst formation have proven responsive to similar alterations in glucose and/or glutamine during culture. Developmental blocks often are associated with the maternal-zygotic transition (MZT) in genomic control (Frei et al., 1989; Telford et al., 1990) and following the MZT, changes in protein synthesis consequently may affect energy metabolism (Rieger, 1992). Prior to the MZT, glucose may alter normal metabolic pathways by promoting glycolysis when the necessary enzymes have not been synthesized (Rieger, 1992) or, alternatively, by acting in concert with inorganic phosphate to accelerate anaerobic glycolysis and inhibit oxidative phosphorylation and ATP production (Seshagiri and Bavister, 1991). In contrast, glutamine may enter the tricarboxylic acid cycle directly so that the glycolytic pathways are bypassed and energy production is supported (Chatot et al., 1990). Studies in other species, such as in mice and cattle have indicated that prior to the MZT, metabolism of glutamine is often high and that following the MZT, glucose metabolism increases substantially (Chatot et al., 1990; Rieger et al., 1992).

In the cat, however, recent limited evidence (Swanson et al., 1994e) suggests that the MZT occurs at the 5- to 8-cell stage *in vivo* and that the morula-to-blastocyst block is more associated with embryo compaction and the transition from the oviductal to the uterine environment. Although the direct effects of glucose may not induce a developmental block

near the expected time of embryonic activation in cats, events associated with glucose metabolism during early culture may have a delayed impact on compaction and blastocoele formation (Ellington et al., 1990). The potential beneficial effects of substituting glutamine for glucose during early culture may be obscured by the requirement for other media factors, not present in mKrb, for blastocyst development.

In a previous study (Johnston et al., 1991a), the percentage of morula developing to blastocyst formation was greater in culture medium (HF-10) supplemented with either 5% FBS (30.8%) or estrual cat serum (22.2%) than with BSA (13.8%) or polyvinyl-alcohol (10.3%), suggesting that unidentified factors in serum promote blastocyst development *in vitro*. In the present study, embryos cultured initially in mKrb containing either glucose or glutamine were switched to HF-10 containing 10% FBS for the final segment of the culture period. Blastocyst formation was enhanced following initial culture in mKrb (glutamine) compared with mKrb (glucose), and percentage of morulae forming blastocysts (22.9%) was similar to those reported by Johnston et al. (1991a), using HF-10 for the entire culture period. These results suggest that, during early culture, glucose in simple medium (mKrb) is contributory to the later morula-to-blastocyst block in cat embryos.

This detrimental effect, however, is not readily apparent when embryos are cultured entirely in HF-10 with FBS, possibly due to other factors in this complex medium that prevent or compensate for the potential adverse effects of glucose. The results also suggested that simple medium, mKrb (glutamine), was equivalent to HF-10 in satisfying the developmental requirements of IVF-derived cat embryos for the first 72 hours of culture, provided that these other unidentified factors are provided later, and indicated that the metabolic requirements of cat embryos are dynamic (i.e., change over time with embryo development). Unfortunately, the results also demonstrated that the optimal conditions for embryo culture likely had not been identified, since so few blastocysts developed during culture.

In the second study, the fertilization rate was lower for oocytes inseminated in HF-10 with 10% FBS (42.6%) than in mKrb with glucose (73.4%) or glutamine (76.7%). This finding was not expected, since Johnston et al. (1991a) have reported greater fertilization (84.0%) rates in HF-10 with 5% FBS. One possible explanation is that different commercial sources of FBS were used in the two studies and/or that the higher percentage of FBS in the present study was inhibitory to embryo cleavage. A study with bovine IVF embryos (Pinyopummintr and Bavister, 1991) indicated that FBS may inhibit the first cleavage division of IVF-derived embryos but without affecting fertilization rates. Because of this low fertilization rate in HF-10 (with FCS), all oocytes for Study III were inseminated in drops of mKrb (glutamine) for 6 h and then transferred to the appropriate culture medium.

The third study was designed to investigate the potential effects of oviductal cell co-culture alone and in conjunction with early culture in mKrb (glutamine). Unexpectantly, results of this study indicated that oviductal cell culture was detrimental to initial embryo cleavage and embryo development to the morula and blastocyst stages. However, culture of embryos initially in mKrb (glutamine) before placement on oviduct cells for the final culture period improved fertilization rate and development to morulae and blastocysts. In cats, sperm capacitation and fertilization post-insemination are reported to require ≤ 5 h in mKrb (Niwa et al., 1985; Goodrowe et al., 1988c) so an inadequate fertilization period was unlikely a cause for the decreased initial embryo cleavage with oviductal cell co-culture.

Possibly, unidentified factors produced by the oviduct cells inhibited initial cleavage or were toxic to a subpopulation of oocytes, but a previous study (Roth et al., 1994a) reported similar fertilization rates with oviduct cells and in medium alone. Nevertheless, these results indicated that oviductal cell co-culture was not beneficial to embryo development, similar to findings reported by Roth et al. (1994a). In the latter study, embryos were fertilized and cultured on a defined oviduct epithelial cell line but there were

no observable differences in development compared with embryos cultured in medium alone. Furthermore, no blastocysts were formed with either treatment. Collectively, the ineffectiveness of co-culture in these two studies was unexpected, since oviduct cell co-culture has consistently overcome earlier developmental blocks in other species, including the sheep (Gandolfi and Moor, 1987), cow (Eyestone and First, 1989), pig (White et al., 1989), sheep (Rexroad and Powell, 1988), goat (Prichard et al., 1992) and rabbit (Carney and Foote, 1990). Perhaps fresh oviduct cells, rather than frozen-thawed monolayers would be more supportive of blastocyst formation, as reported in cattle (Ellington et al., 1990). However, preliminary data from this laboratory (Swanson, unpublished) suggest that there is no discernible difference between fresh or frozen-thawed oviduct cells for the culture of cat embryos.

In the third study, the detrimental effects of oviduct cell co-culture were neutralized by initial culture in mKrb (glutamine), indicating that the adverse impact of co-culture was manifested during the early developmental period. In conjunction with earlier findings concerning mKrb (glucose), this implies that co-culture might eliminate those factors in HF-10 that potentially neutralize the adverse effects of glucose in early culture. However, given the daily replenishment of medium in the culture wells, it would seem unlikely that nutrient or factor depletion would occur with the oviductal cell monolayers.

In conclusion, the morula-to-blastocyst developmental block in IVF-derived cat embryos remains an enigma, occurring at a relatively advanced stage of development and proving resistant to culture alterations that overcome blocks in other species. It is still unknown whether the intractability of the block is a consequence of innate biological defects associated with exogenous gonadotropin-induced folliculogenesis or a result of suboptimal culture conditions during different periods of development, as suggested by the present study. These questions eventually may be resolved by further examination of the *in vivo* developmental capacity of IVF-derived cat embryos as well as by a more systematic investigation of *in vitro* embryo developmental requirements.

CHAPTER VI

TRANSCERVICAL EMBRYO TRANSFER IN THE DOMESTIC CAT

Introduction

Embryo transfer in the domestic cat may play an important role in the reproductive management of laboratory cat colonies. The domestic cat serves as an laboratory animal model for a number of human genetic diseases but, unfortunately, populations of these afflicted cats are often difficult to maintain through natural breeding. Because of inbreeding and physiological abnormalities associated with their disease state, these cats frequently exhibit impaired reproductive function (Howard et al., 1993b). Assisted reproductive techniques, such as artificial insemination, *in vitro* fertilization (IVF) and embryo transfer, may be required to perpetuate many of these genetically valuable cat lineages. In addition to these domestic cat research models, many endangered species of nondomestic felids demonstrate deficient reproduction in captivity and also might benefit from efficient assisted reproductive techniques (Wildt et al., 1992b).

To date, successful embryo transfer in the domestic cat has depended upon surgical transfer procedures (Kraemer et al., 1979; Gruffydd-Jones et al., 1982; Goodrowe et al., 1988a,b; Dresser et al., 1988). These procedures typically require a midventral laparotomy or laparoscopy (A.M. Donoghue, personal communication) for the deposition of the embryos into the lumen of the oviducts or uterus. A nonsurgical embryo transfer technique could offer several advantages over these surgical approaches, including: 1) the elimination of certain potential operative or post-operative complications, 2) a decrease in transfer expense and 3) a reduction in the required level of veterinary expertise. Together, these benefits would possibly increase the utility of embryo transfer for cat propagation purposes.

Transcervical catheterization for nonsurgical embryo collection has been reported in the cat (Hurlbut et al., 1988) but, because of certain anatomical constraints, the technique is inconsistent and penetration of the cervix can not be ensured. The reproductive tract of the

cat is characterized by a tight cervical canal, orientated obliquely between the uterine body and the vagina. The vagina possesses a narrow lumen, further constricted by a prominent dorsal median postcervical fold, and a fornix ventral and lateral to the external cervical opening (Crouch, 1969). The cervix cannot be visualized for catheterization and catheters inserted blindly into the vagina usually are directed ventrally into the vaginal fornix. However, with further definition of these anatomical constraints, it might be possible to design the proper instruments to permit routine catheterization of the cervix. In this study, we describe the development of a transcervical (i.e. nonsurgical) technique for embryo transfer in the domestic cat and the successful application of this technique for the production of viable offspring.

Materials and Methods

Design and Refinement of Embryo Transfer Instruments

Reproductive tracts were obtained from 21 adult female cats, euthanized at the local animal control facility 1 to 2 hours prior to dissection. The reproductive tracts were removed and the lengths of the vestibule (vulva to urethral orifice), vagina (urethral orifice to cervix), uterine body (cervix to uterine bifurcation) and uterine horns (bifurcation to oviduct) were measured (±1 mm). Mean values (± SEM) for the lengths of the combined vestibule/vagina, the uterine body and the uterine horns were calculated. The vestibule and vagina of several representative tracts were injected with clear silicone sealant (Silicone II, General Electric Company, Waterford, NY), covered with moistened tissue (Kimwipes, Kimberly-Clark Corporation, Roswell, GA) and maintained in a refrigerator (5°C) overnight to permit hardening of the silicone. The next day, the reproductive tracts were bisected longitudinally and the silicone impression molds were removed.

The reproductive tract dimensions and the impression molds were used in the design of a form-fitting vaginal speculum and a transcervical catheter (Figure 12). A vaginal speculum was fashioned from a cut section (80 mm) of an 8 Fr. polypropylene

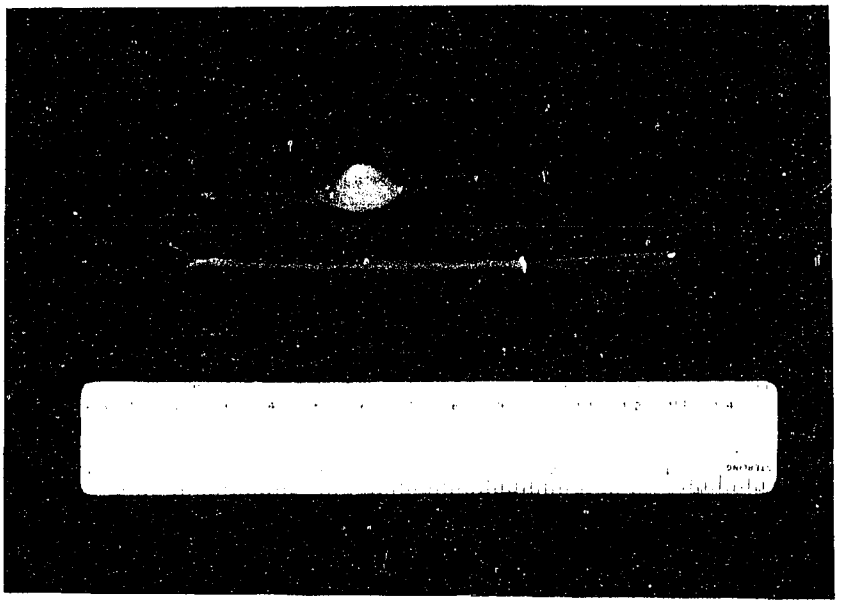

Figure 12. (a) Silicone impression mold of domestic cat vagina and vestibule and (b) vaginal speculum, transcervical catheter and embryo transfer tubing.

urinary catheter (Sovereign, Monoject, Division of Sherwood Medical, St. Louis, MO) by gently heating one end of the section over a propane burner and molding the softened plastic into the desired shape, using the silicone impression mold as a guide. The molded plastic was trimmed with a scalpel blade and smoothed with fine grain sand paper to produce a contoured distal end, with the distal opening of the speculum positioned 3 mm from the tip. The outer diameter (o.d.) of the speculum (2.7 mm) was set at the maximal size that would fit into the vaginal lumen of most cats while the length of the speculum (80 mm) was designed to permit complete vaginal insertion with ~30 mm of speculum protruding externally.

The transcervical catheter was made from a 3.5 Fr. (1.2 mm o.d.; 140 mm length) open end polypropylene urinary catheter (Sovereign, Monoject, Division of Sherwood Medical) by cutting 27 mm off the distal end, gently heating and bending the tip (10 mm) at a 30° angle and finally sanding smooth any sharp edges. Lastly, embryo transfer tubing (0.28 mm inner diameter; 0.61 mm o.d.; 200 mm length) was fashioned from polyethylene tubing (PE 10, Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ), connected to a blunted 30 gauge hypodermic needle on a 1 ml tuberculin syringe. Using excised reproductive tracts and tracts *in situ* in deceased cats, preliminary speculum and catheter designs were evaluated for functionality and gradually modified to arrive at the final instrument specifications.

In Vitro Fertilzation and Embryo Culture

To demonstrate the utility of the transcervical technique, a series of four transfer trials were conducted, using IVF-derived embryos. Random-source, adult male and female cats were housed singly or in pairs in stainless steel cages (1 x 1 x 1 m) and maintained under controlled fluorescent lighting (12 hours light:12 hours dark). Cats were provided a commercial feline diet (Science Diet, Hills Pet Products, Topeka, KS) and water *ad libitum* and all cats were maintained in accordance with Institutional Animal Care and Use Committee guidelines. To generate pre-implantation embryos for transfer trials (and

embryo developmental studies), IVF and embryo culture were performed on four occasions following an established protocol (Goodrowe et al., 1988a; Johnston et al., 1991a,b). Briefly, anestrual queens (n=2, trial 1; n=6, trial 2; n=5, trial 3; n=3, trial 4)) were injected i.m. with 150 IU equine chorionic gonadotropin (eCG; Sigma Chemical Company, St. Louis, MO), followed 84 hours later with an injection of 100 IU. human chorionic gonadotropin (hCG; Sigma). Ovarian follicles were aspirated at laparoscopy 24-27 hours following the hCG injection and recovered mature oocytes were inseminated in microdrops (100 µl) of modified Kreb's Ringer bicarbonate medium (Johnston et al., 1991a; Toyoda and Chang, 1974) (prepared from stock chemicals; Sigma) with sperm cells (2 x 10^4 motile) collected by electroejaculation of a fertile male. At 30 hours post-insemination, fertilization was assessed as cleavage to at least the 2-cell stage and embryos designated for transfer trials were cultured in 100 µl microdrops of Ham's F-10 medium (Sigma), supplemented with 10% fetal bovine serum (FBS), under oil. Embryos were cultured *in vitro* (38 °C; 5% CO_2 in air) an additional 4 or 5 days to the morula stage of development prior to transfer.

Embryo Transfer

Embryo recipients (n=8) were either oocyte donors (n=2, trial 1; n=2, trial 2; n=1, trial 3) or queens in natural estrus (n=3, trial 4), induced to ovulate with two 25 µg intramuscular injections of gonadotropin releasing hormone (GnRH)(Cystorelin, Ceva Laboratories, Overland Park, KS) administered 12 hours apart. For transfers, recipients were anesthetized with 8 mg/kg of tiletamine-zolazepam hydrochloride (Telazol, A.H. Robins Company, Richmond, VA) at 6 or 7 days post-hCG (oocyte donor recipients) or 6 days post-GnRH (natural estrus recipients). Recipients were administered 25,000 U/kg of penicillin i.m. (Procaine Penicillin G, Pfizer, New York, NY) after induction of anesthesia. Based on previous reports (Goodrowe et al., 1988a; Donoghue et al., 1992b), oocyte donor recipients were assumed to possess functional corpora lutea (CL) several days post-aspiration. However, to correct for a possibly altered endocrine environment, the first four

oocyte donor recipients were supplemented i.m. with 5 mg progesterone in oil (Progesterone Injection, United Research Laboratories, Philadelphia, PA) at the time of transfer. To verify that ovulation had occurred in the natural estrus recipients in response to the GnRH injections, these queens were subjected to laparoscopy just prior to embryo transfer.

For embryo transfer, each recipient was placed in sternal recumbency with her tail secured cranially and her hind end slightly elevated. A lightly lubricated vaginal speculum was directed dorsally through the vestibule into the vagina and inserted slowly until resistance prevented further penetration. A sterile transcervical catheter was inserted to the end of the speculum and gently advanced through the external cervical os. Sterile transfer tubing, without embryos, was passed through the catheter and advanced up into a uterine horn. Minimal resistance on catheter placement and on tubing advancement were considered indicative of proper positioning of the speculum and catheter, respectively. After transcervical catheterization was assured, embryo transfer was performed using IVF-derived morulae. Embryos, in 10 µl Ham's F-10 medium (with 10% FBS), were drawn up into sterile transfer tubing, passed through the catheter and deposited, 80-175 mm from the vulva, into the lumen of one uterine horn. At 20-25 days post-transfer, recipients were subjected to abdominal palpation and ultrasonography to determine pregnancy status.

Results

Based on the measurements of the excised reproductive tracts, the mean values (± SEM) for the lengths of the combined vestibule/vagina, the uterine body and the uterine horns were calculated to be 49.9 ± 1.1 mm, 31.9 ± 1.0 mm and 72.5 ± 2.8 mm, respectively. Using these measurements, the optimal dimensions for the transfer instruments were determined. The form-fitting vaginal speculum was designed to fit snugly against the cervix and align the distal opening of the speculum with the external cervical os (Figure 13). The semi-rigid transcervical catheter, with its slight distal

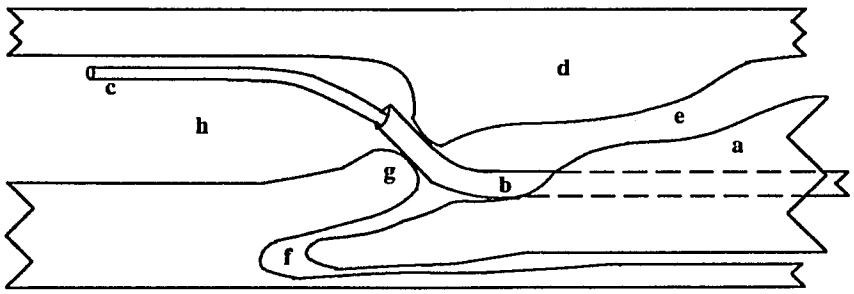

Figure 13. Transcervical embryo transfer technique: The form-fitting vaginal speculum (a) aligns the transcervical catheter (b) with the cervical canal (g), permitting the deposition of embryos, via transfer tubing (c), into the uterus (h). dorsal median postcervical fold (d), vagina (e), ventral vaginal fornix (f)

angulation, was shaped to easily enter and penetrate the cervical canal and to terminate 5-10 mm into the uterine lumen. With proper placement of the transcervical catheter, the transfer tubing could be advanced up into the uterine horns with minimal resistance. With cat cadavers as practice models, the final instrument design consistently permitted transcervical catheterization and transfer tubing advancement into the uterine horns.

With laparoscopic follicular aspiration, 257 mature oocytes were recovered from the oocyte donors (n=16) and, following IVF, 182 embryos were generated (70.8% fertilization rate). Two- to 4-cell embryos (n=80) were randomly assigned to the transfer study and, following embryo culture, the resulting morulae (n=46) were transferred in a series of four trials (Table 9). With most recipients, the transfer instruments were easily placed in their proper positions and the actual transfers were uneventful. For one natural estrus recipient (Cat No. 6), however, the vaginal speculum could not be fully inserted and the transfer attempt was aborted.

Pregnancy was detected by ultrasound in one natural estrus recipient (Cat No. 7) at 22 days post-transfer. This female subsequently gave birth to three full-term kittens (2 healthy, 1 stillborn) at 61 days post-transfer (Figure 14). Pregnancy was not diagnosed in any other recipient. No post-transfer complications were observed in any of the embryo recipients.

Discussion

The results of this study have demonstrated that transcervical catheterization is feasible in the domestic cat and that the technique may be useful as a potential alternative to surgical embryo transfer procedures. The described transfer technique is a nonsurgical procedure that requires little equipment, veterinary expertise or expense to perform. The vaginal speculum and transfer catheter can be easily made by modifying commercially available polypropylene urinary catheters and can be repeatedly sterilized by steam autoclave without losing shape. By adapting the vaginal speculum and transcervical

Table 9. Summary of embryo recipient status, embryo characteristics and transfer results for transcervical embryo transfer procedures in domestic cats

Trial no.	Cat no.	Recipient status[a]	Progesterone (yes/no)[b]	Embryo culture duration (days)	No. embryos transferred[c]	Pregnancy (yes/no)[d]
1	1	oocyte donor	yes	6	6	no
	2	oocyte donor	yes	6	7	no
2	3	oocyte donor	yes	5	4	no
	4	oocyte donor	yes	5	5	no
3	5	oocyte donor	no	5	6	no
4	6[e]	natural estrus	no	-	-	-
	7	natural estrus	no	5	9	yes
	8	natural estrus	no	5	9	no

[a] Oocyte donor recipients were stimulated with eCG/hCG and ovarian follicles were aspirated 5 or 6 days prior to embryo transfer while natural estrus recipients were induced to ovulate with GnRH administered 6 days prior to embryo transfer.

[b] Recipients supplemented (i.m.) with 5 mg progesterone in oil.

[c] All embryos were morulae at the time of transfer.

[d] Pregnancy status determined by palpation and ultrasonography 20-25 days post-transfer.

[e] Transcervical catheterization was not possible and no embryos were transferred.

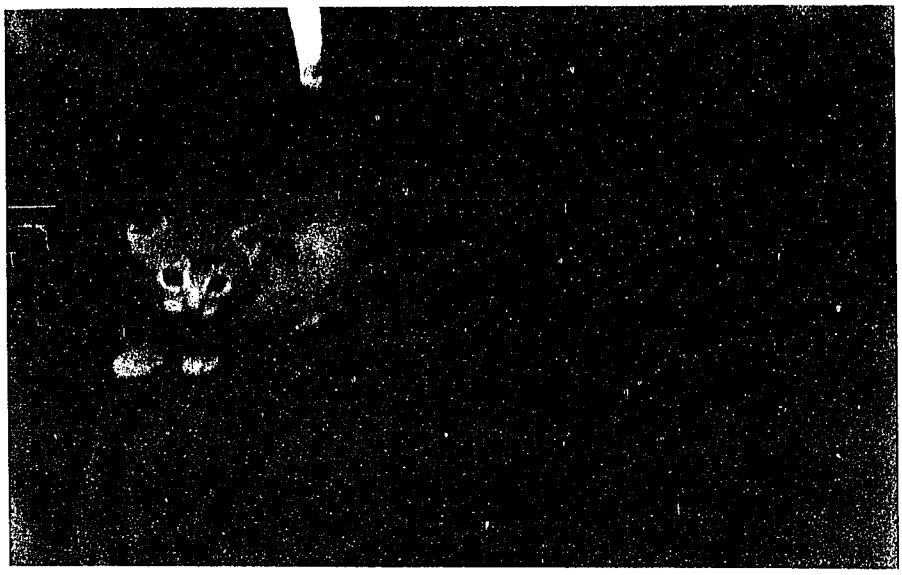

Figure 14. Domestic cat kittens born following transcervical transfer of IVF-derived embryos

catheter to conform to the reproductive anatomy of the cat, the cervix can be consistently cannulated in most cats, providing a reliable method for intrauterine embryo deposition. A few recipients, however, may have a narrow vaginal lumen that will not accommodate the standard-sized (8 Fr.) speculum and a smaller diameter speculum may be required.

The overall efficiency of this technique, and cat embryo transfer in general, likely will improve when the effects of certain variables affecting embryo survival are resolved. For example, the type (natural or induced cyclicity) and degree of synchrony of the recipient and the nature (*in vivo*- or *in vitro*-derived) and stage of development of the embryos may influence the success of embryo transfer. Recipients stimulated with exogenous gonadotropins and subjected to ovarian aspiration rapidly form functional CL (Goodrowe et al., 1988a; Donoghue et al., 1992b) but also may develop ancillary follicles (Donoghue et al., 1992b) and secondary CL several days post-aspiration (Goodrowe et al., 1988a), possibly causing abnormal hormone ratios (estradiol:progesterone) during the early luteal phase. For this reason, the first four recipients, all oocyte donors, were supplemented with injections of progesterone. This strategy has proven useful in domestic cats (Dresser et al., 1988; Donoghue, personal communication) and tigers (Donoghue et al., 1990) but, in this study, no pregnancies resulted in the progesterone-supplemented domestic cats. Serum hormone concentrations were not determined but the dosage and timing of progesterone supplementation may have been inappropriate, producing a temporal excess in progesterone levels with adverse effects on embryo survival.

In addition to recipient status, the nature of the transferred embryos may affect the transfer success. The viability of IVF-derived embryos, especially at later stages of development, may be compromised relative to embryos generated *in vivo*. Typically, IVF-derived embryos stop developing at the morula stage after 4-5 days *in vitro* and then undergo degeneration (Johnston et al, 1991a). In the present study, a single pregnancy occurred following the transfer of IVF-derived embryos, after a relatively short culture period, to a natural estrus recipient. These results suggest that post-transfer embryo

survival might be improved by limiting the duration of *in vitro* embryo culture to 5 days and by utilizing only naturally cycling females as recipients.

In this study, the ovaries of the natural estrus recipients were examined laparoscopically prior to embryo transfer to verify that ovulation had occurred. Alternately, ovulation and CL function can be assessed using a rapid progesterone assay (Target Rapid Feline Progesterone Kit, BioMetallics, Princeton, NJ) for blood serum (Hammer, 1993) or plasma. With the aid of this assay, the presence and functionality of luteal tissue could be determined in less than 20 minutes for all embryo recipients and no surgical procedures are necessary.

This nonsurgical embryo transfer procedure in cats has several potentially important applications. For domestic cat lineages that serve as models for human genetic diseases, refined embryo transfer and other assisted reproductive techniques may help alleviate their impaired reproductive capacities (Howard et al., 1993b). *In vitro* fertilization procedures may be used to generate embryos from afflicted cats and the embryos then could be nonsurgically transferred to healthy, synchronized recipients. Alternately, these embryos may be cryopreserved and transferred at a later time. In conjunction with routine embryo cryopreservation protocols (Dresser et al., 1988; Pope et al., 1993b), this simple, noninvasive embryo transfer procedure would eliminate the necessity for surgical transfers while promoting the distribution of valuable cat embryos among laboratory animal facilities.

Although the technique was developed for embryo transfer purposes, the vaginal speculum and transcervical catheter may have additional uses in other areas of assisted reproduction and veterinary medicine. Specifically, transcervical catheterization may facilitate the development of nonsurgical techniques for intrauterine artificial insemination and of more efficient nonsurgical procedures for embryo collection. In addition, with transcervical catheterization, intrauterine infusions of antibiotic lavage solutions for conditions such as endometritis or pyometra may be more feasible as a therapeutic option

(Vasseur and Feldman, 1982). Lastly, with some modification of the speculums and catheters, this nonsurgical embryo transfer procedure may have similar utility with endangered nondomestic cat species. A nonsurgical technique would minimize the stress and procedural risk of embryo transfer in these species and, consequently, may increase the number of zoos willing to participate in these critical reproductive studies.

SUMMARY AND CONCLUSIONS

In a series of five studies, immunological and developmental factors affecting the efficacy of *in vitro* fertilization (IVF), embryo culture and embryo transfer (ET) were investigated in the domestic cat. These studies focused on four primary concerns with these assisted reproductive techniques, specifically 1) the immunological consequences of exogenous gonadotropin treatment of cats to induce ovarian stimulation; 2) the potential effects of exogenous gonadotropin regimens upon the maternal environment in ET recipients; 3) the developmental requirements of IVF-derived cat embryos and relevance to their biological competence and 4) the requirement for surgical procedures of ET.

In the first study, domestic cats were treated with combination regimens of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to induce ovarian stimulation as a component of an established IVF protocol. Cats exhibited decreased ovarian responsiveness following the repeated administration of eCG/hCG at short intervals (44-50 days) and a possible immunological mechanism for this ovarian refractoriness was evaluated. Using an enzyme-linked immunoabsorbent assay (ELISA), sera from male, naive female and previously eCG/hCG-stimulated (1x, 3x, 4x) female cats were analyzed for the presence of eCG, hCG and porcine follicle stimulating hormone (pFSH) -binding immunoglobulins. Sera of cats receiving multiple eCG/hCG injections, at short intervals, displayed a greater level of eCG-binding activity than sera of male, naive female or singly-stimulated female cats and demonstrated variable affinity for hCG and/or pFSH.

In pre-ovulatory and post-ovulatory mouse ovarian stimulation assays, mice injected with an eCG/antisera mixture had lower ovary weights than mice injected with eCG/saline and decreased numbers of ovulated oocytes compared to mice treated with eCG/naive sera. Treatment of eCG/hCG refractory queens with a pFSH/hCG regimen caused a rebound in ovarian follicular development but not in oocyte maturity. The results of this study indicated that repeated treatment of domestic cats with eCG and hCG may

cause an immunologically-mediated refractoriness to ovarian stimulation. Although alternative gonadotropin regimens may alleviate this refractoriness, it was concluded that a preferable strategy might be the avoidance of potential immunological complications through the judicious use of eCG and hCG in domestic and nondomestic felids.

In the second study, the ovarian responses and kinetics of gonadotropin-binding immunoglobulin production were investigated in domestic cats repeatedly injected with eCG and hCG at short (49-57 days) or long (130-135 days) treatment intervals. Queens were treated three or four times with a standard eCG/hCG regimen and subjected to laparoscopy following each treatment to evaluate ovarian follicular development. Serial serum samples were assessed by ELISA for the presence of eCG-binding immunoglobulins. Queens repeatedly stimulated with eCG/hCG at the longer intervals typically showed no decrease in ovarian follicle production or in maturity of recovered oocytes while queens treated at the shorter interval exhibited reduced follicular development and compromised oocyte maturity by the third stimulation. For both interval groups, ELISA data indicated individual variability in seroconversion following eCG/hCG challenge.

In general, peak immunoglobulin titers were higher for queens stimulated at the short intervals compared with queens in the long interval group and high serum titers at the time of eCG/hCG injection or rapid increases in titers immediately post-injection were associated with poor ovarian responses. The results of this study indicated that both individual variability in immune responses and the interval between repeated gonadotropin stimulations are critical factors for determining if queens will develop immunologically-mediated ovarian refractoriness. These results also suggested that intervals between eCG/hCG treatments of at least 4 months should be recommended for assisted reproductive procedures in domestic and nondomestic cats.

In the third study, the potential suitability of the maternal environment was investigated in queens treated with the standard eCG and hCG combination regimens. The rate of elimination of eCG from circulation was estimated and, following follicular

aspiration, the formation of ancillary follicles and secondary corpora lutea (CL) was characterized. The effect of gonadotropin-neutralizing antisera on the development of secondary ovarian structures, CL function and humoral immune responses also was evaluated.

After intramuscular injection, initial serum eCG concentrations were variable, with the elimination half-life estimated at 24-48 hours and with eCG persisting in circulation for several days. Following follicular aspiration, queens formed CL equal to the number of aspirated follicles and exhibited a rapid increase in progesterone concentration but developed high numbers of ancillary follicles by 5 days post-aspiration. By 15 days post-aspiration, all ancillary follicles had luteinized to form secondary CL. Treatment with neutralizing antisera at the time of follicular aspiration slowed primary CL formation but did not decrease the number of ancillary follicles or secondary CL. Progesterone concentrations did not differ from control queens while secondary humoral immune responses to eCG were qualitatively similar between groups. These results indicated that eCG was eliminated slowly from cats following intramuscular injection and this persistence in circulation may have contributed to the development of ancillary follicles and secondary CL. However, the administration of neutralizing antisera at the time of follicular aspiration was ineffective in preventing the formation of these secondary ovarian structures.

In the fourth study, a series of three experiments were conducted to examine the influence and temporal interaction of different energy substrates, media complexities and tissue co-culture on the development of *in vitro* fertilized cat embryos and the persistence of the morulae-to-blastocyst developmental block. In the first experiment, oocytes were fertilized in a simple culture medium, modified Krebs Ringer bicarbonate (mKrb), containing either glucose or glutamine, and then cultured in the same medium for 144 hours or in mKrb (glutamine) for the first 72 hours followed by mKrb (glucose) for the final 72 hours. Fertilization rate, percent development to morulae and cell number/embryo did not differ between treatments and blastocyst formation was low (<10%) for all treatments.

In the second experiment, oocytes were fertilized in mKrb (with glucose or glutamine) or a complex medium, Ham's F-10 (HF-10; supplemented with 10% fetal bovine serum , FBS), and cultured for 144 hours in HF-10 or in mKrb (glucose) or mKrb (glutamine) initially (for 72 hours) and then HF-10 for the final 72 hours. Fertilization rate was lower in HF-10 but embryo development to the morula stage was comparable for all treatments. Embryos cultured in mKrb (glutamine)/HF-10 exhibited a greater cell number per embryo, percentage of blastocysts and percentage of morulae forming blastocysts compared to embryos cultured in mKrb (glucose)/HF-10.

In the third experiment, all oocytes were fertilized in mKrb (without glucose or glutamine) and resulting embryos were cultured for 72 hours in mKrb (glutamine) and then switched to either HF-10 or cat oviductal cell monolayers (in HF-10) for the final 72 hours. Additional embryos were co-cultured for the entire period in HF-10 or on cat oviductal cell monolayers. Fertilization rates were lower on oviduct cells than other treatments but cell number per embryo was similar in all treatments. Blastocyst formation percent was lower on oviduct cells than in mKrb (glutamine)/HF-10 treatment and was < 20% in all treatments. These results indicated that while IVF-derived cat embryos developed to morulae under a variety of culture conditions, the morula-to-blastocyst developmental block is resistant to alterations of energy substrate and medium complexity or fluctuations in their temporal availability. In addition, the presence of glucose during the initial culture period appeared to be detrimental to later blastocyst formation, and oviduct cell co-culture, alone or in combination with other culture variations, was ineffective in overcoming the developmental block.

In the final study, the feasibility and potential application of a transcervical ET technique was investigated. Using cadaver models and silicone impressions of cat reproductive tracts, a form-fitting vaginal speculum and transcervical catheter were designed. The application of the technique was evaluated in a series of ET trials, using IVF-derived embryos and either eCG/hCG-treated queens (n=5) or natural estrus/GnRH-

treated queens (n=3) as recipients. Pregnancy was established in one natural estrus/GnRH-treated queen, and 61 days post-transfer, a litter of three kittens was born. The results of this study demonstrated the feasibility of transcervical ET in cats but suggested that embryonic and maternal concerns must be resolved before higher embryo survival and pregnancy rates can be obtained with this approach.

In conclusion, these studies have addressed various immunological and developmental concerns associated with the use of assisted reproductive techniques in felid species but certain problems remain unresolved. The results of the immunological studies indicated that although cats are immunoresponsive to exogenous gonadotropins and can become refractory to ovarian stimulation after repeated treatment, strategies are available to avoid these undesirable immunological consequences. Specifically, prolonging the interval between successive gonadotropin regimens appears to be effective in limiting the production of anti-gonadotropin immunoglobulins but the potential consequences of unlimited treatment at this longer interval are not known. Of additional concern, the individual variability in immune responses in domestic cats indicates that some cats might become refractory even with long treatment intervals.

In regard to nondomestic species, immunological responses to exogenous gonadotropins have not been adequately investigated. Although the domestic cat is considered a suitable animal model for most felid reproductive studies, extreme variability in immune responses to exogenous gonadotropins between species (as with individuals within a species) is a definite possibility and would limit the extrapolation of immunological data from domestic cats.

The effectiveness of alternative gonadotropins in stimulating ovarian follicular development in refractory queens suggests another strategy for avoiding gonadotropin resistance. Provided that immunological crossreactivity is minimal between the specific gonadotropins, different ovarian stimulation regimens might be used in an alternating fashion, possibly at much shorter treatment intervals, but, again, individual/species

variability must be a consideration. In addition, relatively nonimmunogenic, nongonadotropin compounds (such as GnRH) might be incorporated into protocols to limit immunoreactivity to stimulation regimens and to reduce the possibility of the synergistic immunological effects suspected with combination gonadotropin regimens. Obviously, additional research is needed in both domestic and nondomestic cats to investigate these options.

Beyond immunological considerations, the effects of exogenous gonadotropins on the maternal environment still must be a primary concern. Although unequivocal evidence is lacking, the results of the present study and of previous investigations strongly suggest that the maternal environment of exogenous gonadotropin-treated queens is not physiologically normal and is probably unsuitable for supporting embryo/fetal development. Strategies to correct for these possible deficiencies hinge on either mitigating the adverse effects of current synchronization protocols, as attempted with anti-gonadotropin treatment, or developing new protocols that generate a more physiological response. As with immunological considerations, additional studies are needed to further characterize the maternal environment in gonadotropin-stimulated queens and to investigate alternative strategies for recipient synchronization.

Of additional concern, the results of the embryo culture studies support the contention that most IVF-derived cat embryos might not be biologically competent. Even under the most optimal of culture conditions, less than 20% of IVF-derived cat embryos are capable of developing to the blastocyst stage. In contrast, a high percentage (~70%) of *in vivo*-derived embryos are capable of both forming blastocysts *in vitro* and developing to term *in vivo*. These findings might suggest intrinsic abnormalities in many IVF-derived embryos, possibly at the genomic level. However, the true biological competence of IVF-derived embryos is difficult to determine without comparative developmental studies of these embryos *in vivo* (i.e., following ET). Unfortunately, these investigations are confounded by our poor understanding of the factors affecting the maternal environment in

cats. Questions about the adequacy of the maternal environment in cats must be addressed before the developmental capacity of IVF-derived embryos can be thoroughly investigated.

If the issues of embryo competence and the maternal environment are ever adequately resolved, the efficacy of ET procedures in cats would likely improve. For ET in cats, a nonsurgical (transcervical) technique has proven feasible as an option to surgical methods. In the future, as transfer procedures become more efficient, transcervical catheterization may become the preferred method for intrauterine ET in domestic cats. Because of the similarity of reproductive anatomy between domestic and nondomestic cats, extrapolation of this technique to the conservation of endangered felid species should present few difficulties. In closing, the results of the studies of this dissertation indicate that a tremendous amount of additional research is needed in both domestic and nondomestic cats before *in vitro* fertilization, embryo culture and embryo transfer can truly be considered a practical reproductive strategy in cats. However, the potentially invaluable benefits of this strategy, especially in the conservation of endangered nondomestic felids, necessitates its continued refinement as a reproductive option.

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VITA

William Frederick Swanson was born to Peter and Hildy Swanson in Big Spring, Texas on August 28th, 1960. In the first ten years of his life, he and his family lived in Texas, France, South Carolina and California before returning to Texas to permanently reside. In 1978, he graduated from L.C. Anderson High School in Austin, Texas and entered the University of Texas at Austin for undergraduate studies. In 1982, he graduated from the University of Texas with a Bachelor of Science degree in zoology but remained at the university for an additional year to begin graduate studies. He also worked for a year as the assistant kennel manager at the Austin Humane Society before entering the College of Veterinary Medicine at Texas A&M University in 1984 to pursue a veterinary degree. After receiving his Doctor of Veterinary Medicine degree in 1988, he accepted a Board of Regents Graduate Fellowship in Animal Science at Louisiana State University. For the past two years, while writing his dissertation, he has been employed as a reproductive physiologist at the National Zoological Park in Washington D.C. He plans to continue his research into the conservation of endangered nondomestic felids upon the completion of this degree.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: William Frederick Swanson

Major Field: Animal Science (Reproductive Physiology)

Title of Dissertation: Immunological and Developmental Factors Affecting the Efficacy of In Vitro Fertilization, Embryo Culture and Embryo Transfer in the Domestic Cat

Approved:

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